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The Jun family Members, c-Jun and JunD, Transactivate the Human c-*myb* Promoter via an Ap1-like Element*

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The c-*myb* protooncogene, which is preferentially expressed in hematopoietic cells at the G_1/S boundary of the cell cycle, encodes a transcriptional activator that functions via DNA binding. The regulatory mechanisms governing this specific pattern of expression are not fully understood, although human c-*myb* expression appears to be positively autoregulated via myb-binding sites in the 5′-flanking region of the c-*myb* gene (Nicolaides, N. C., Gualdi, R., Casadevall, C., Manzella, L., and Calabretta, B. (1991) *Mol. Cell. Biol.* 11, 6166–6176). To determine the contribution of other transcription regulators such as JUN family members in the control of c-*myb* expression, transient expression assays were carried out which revealed a 6- to a 15-fold enhancement by c-Jun and JunD, but not JunB, in chloramphenicol acetyltransferase reporter gene expression driven by different segments of the human c-*myb* 5′-flanking region. An Ap1-like element located at nucleotide −149 from the c-*myb* initiation site appears to be required for this transactivation upon binding to a nuclear protein complex containing c-Jun and JunD, since site-directed mutations of this Ap1-like element abolished c-Jun and JunD binding and transactivation. Exposure of phytohemagglutinin-stimulated peripheral blood mononuclear cells to c-*jun* and junD antisense oligodeoxynucleotides resulted in a 46 and 43% inhibition of T-lymphocyte proliferation that was accompanied by a decrease in c-*myb* mRNA levels as compared with sense-treated cultures. Because T-lymphocytes induced to proliferate express c-*jun* and junD before c-*myb*, these data suggest a mechanism whereby c-Jun and JunD contribute to the transcriptional activation of c-*myb* that, in turn, is maintained at the G_1/S transition and during S phase by positive autoregulation.

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). The restricted ability of the v-*myb* containing avian myeloblastosis and E26 viruses to transform hematopoietic cells is paralleled by a predominant pattern of c-*myb* expression in immature hematopoietic tissues, which has long suggested that the function of c-*myb* is restricted to immature hematopoietic cells (3, 4). In activated normal T-lymphocytes, down-regulation of c-*myb* protein synthesis prevents the G_1/S transition (5–7). Most recently, introduction of a constitutively expressed c-*myb* cDNA into murine Balb/c3T3 fibroblasts was shown to abrogate the requirement for IGF-1 further suggesting that c-*myb* plays an important role in cell cycle regulation acting at a critical point preceding entry into S phase (8).

Myb proteins have several properties characteristic of transcriptional regulators, including nuclear localization (9, 10) DNA binding ability *in vitro* (11, 12), and transcriptional activation and repression functions (13), suggesting that the regulatory effect on the G_1/S transition is exerted at the transcriptional level. The DNA-binding domain recognizes the consensus sequence pyAAC(G/T)G (14). Subsequent experiments have demonstrated Myb binding to variants of this consensus sequence (15–17), although the pyAAC sequence appears to be the major element needed for high affinity Myb binding. In transient assays, Myb binding to reporter constructs that included Myb-binding sites has clearly been shown to enhance the expression of the reporter gene regardless of the orientation of the binding sites (17–19). Only a few promoters (the avian MIM-1 (18), SV40 (15), human c-*myb* (17), etc.) have been clearly shown to contain functional Myb-binding sites. These promoters contain three or four closely spaced Myb-binding sites, a feature that might be significant in c-*myb*-regulated promoters because multiple binding sites are known to increase gene expression (18).

Sequence analysis of the human c-*myb* 5′-flanking region has revealed the presence of multiple GC boxes which appear to confer a basal level of promoter activity to the c-*myb* 5′-flanking region and multiple Myb-binding sites which appear to be involved in positive autoregulation of c-*myb* expression in cell types with detectable c-*myb* mRNA levels. This autoregulation of c-*myb* expression might, in turn, amplify the mitogenic signal encoded by c-*myb* to allow the interaction of Myb protein with regulatory elements of genes required at the G_1/S transition or during S phase. However, this simple model does not take into consideration the contribution of other transacting factors in c-*myb* regulation of the G_1/S transition. For example, it is possible that c-*myb* expression is regulated by transcriptional factors that interact with regulatory elements of the gene and that up-regulate its expression over a threshold that allows its positive autoregulation. To test this possibility, we examined whether members of the Jun gene family (c-Jun, JunB, and JunD) which are "early G_1" response genes and presumably function before c-*myb* are involved in the regulation of the c-*myb* expression. Our data indicate that c-Jun and JunD transactivate c-*myb* expression

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via a Jun-binding site in the 5′-flanking region of the human c-*myb* gene.

MATERIALS AND METHODS

Plasmids—Reporter constructs were made by linking different segments of the human c-*myb* promoter to the bacterial chloramphenicol acetyltransferase (CAT)[1] gene contained within 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 pE-3, which contains a *Bam*HI-*Eco*RI fragment of the human c-*myb* gene from nt −687 to +307 including 687 bp of the 5′-flanking region, the first exon, and part of the first intron. The pE-3 plasmid was cloned in the Bluescript SK vector in the sense orientation to the T3 primer. The B1CAT plasmid, which contains nt −687 to +204 of the c-*myb* gene, was constructed by ligating a blunt-ended 0.89-kilobase *Bam*HI-*Nco*I fragment from the pE-3 plasmid into a blunt-ended *Sal*I site in the polylinker of the pUCCAT construct in the sense orientation to the CAT gene. The S1CAT plasmid, which contains nt −197 to +204 of the c-*myb* gene, was constructed by removing an *Eco*RV-*Pst*I fragment (nt −687 to −78) from B1CAT and ligating to a *Sma*I-*Pst*I fragment (nt −197 to −78) from the pE-3 plasmid. The P1CAT plasmid, which contains a c-*myb* gene fragment from nt −78 to +204, was constructed by removing a 0.63-kilobase *Pst*I fragment from the B1CAT plasmid and religating the vector. Constructs used in the mutagenesis assays were derived as follows: 1) pJD1CWTCAT was made by digesting pJUND1CWT (see below) with *Eco*RI, blunt-ended by filling with Klenow enzyme, and then digesting with *Hin*dIII. This 270-bp *Eco*RI (blunt) *Hin*dIII fragment was gel purified and cloned into the blunted *Sal*I (filled in with Klenow) *Hin*dIII site of the pUCCAT vector. 2) pJD1CMUTCAT was made as described for pJD1CWTCAT except that the insert fragment was from the plasmid p2JUND1CMUT (see below). pSV2CAT was used as a positive control which consists of the CAT gene driven by the SV40 early promoter and enhancer and polyadenylation signal.

The jun effector plasmids were constructed by cloning the murine c-*jun*, junB, and junD cDNAs into the pSV40 polylinker (pSV) plasmid which contains the early promoter and polyadenylation signal of simian virus 40 (SV40). The pSVc-jun was made by cloning an *Eco*RI-*Xho*I fragment containing the c-*jun* cDNA into the *Eco*RI-*Xho*I site of the pSV plasmid. pSVjunB was made by cloning an *Eco*RI-*Xho*I fragment containing the junB cDNA into the *Eco*RI-*Xho*I site of the pSV plasmid. The pSVjunD was made by cloning an *Eco*RI fragment containing the junD cDNA into the unique *Eco*RI site of the pSV plasmid. All effector plasmids were shown to express high levels of RNA (by Northern blot analysis) and protein (by Western blot analysis) (data not shown). The c-*myb* effector plasmid was the previously described pMbmI (17) in which the human c-*myb* full-length cDNA is driven by the SV40 early promoter.

Plasmids used for *in vitro* transcription experiments were constructed as follows: 1) pSKjunB, the junB cDNA *Eco*RI-*Xho*I fragment was cloned into the Bluescript SK *Eco*RI-*Xho*I site in the sense orientation to the T3 primer; 2) pSKc-jun, the c-*jun* cDNA *Eco*RI-*Pst*I fragment was cloned into the *Eco*RI-*Pst*I site in SK in the sense orientation to the T7 primer; and 3) pG3ZjunD, the junD cDNA *Eco*RI fragment was cloned into the *Eco*RI site of the PGEM3Z plasmid (Promega, Madison, WI) in the sense orientation to the T7 primer.

Site-directed Mutagenesis—Site-directed mutations were introduced in the JunD-binding site centered at position −149 of the human c-*myb* promoter by polymerase chain reaction (PCR) as described (17). PCR was done using a mutated 5′-oligonucleotide as primer, corresponding to nt −164 to −142 and changing the wild-type sequence 5′-AATGGGAGCGACGACCCGGCCA-3′ to 5′-AATGGGAGAAACTTCCCGGCCA-3′, and a 3′ primer corresponding to nt +80 to +60. PCR was done using 1 ng of plasmid pE-3. Amplification conditions were 1.5 min at 94 °C, 1.5 min at 45 °C, and 2.0 min 72 °C, for 20 cycles. The same amplification was done using a wild-type 5′-oligodeoxynucleotide from −164 to −142. PCR products were then run on a 2% agarose gel, purified using Gene Clean II (Bio101, LaJolla, CA), and cloned into the PCR2000 cloning vector (InVitrogen, San Diego, CA). Sequence analysis revealed clones that were in the sense orientation to the T7 primer. The wild-type recombinant was designated p2JUN1CWT, and the mutant recombinant, was p2JUN1CMUT.

Transient CAT Analysis—CAT assays were performed as described (20). Briefly, 5 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 Tk-ts13 Syrian hamster fibroblasts (21) using the calcium-phosphate precipitation method (22). 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 C^{14}-labeled chloramphenicol and acetyl-CoA for 1 h at 37 °C. Transactivation of reporter constructs was assayed by measuring the amount of acetylated [^{14}C]chloramphenicol by thin layer chromatography followed by autoradiography and Cherenkov counting.

In Vitro Transcription and Translation—Plasmid DNAs were isolated by the polyethylene glycol method (23). To generate sense mRNAs, templates were linearized with unique restriction enzymes 3′ to the cDNA (junB with *Xho*I, c-*jun* with *Sac*I, and junD with *Sac*I). To generate antisense mRNAs, templates were linearized with unique restriction sites 5′ to the cDNA (junB with *Eco*RI, c-*jun* with *Hin*dIII, and junD with *Nde*I). Capped mRNAs were synthesized using the appropriate RNA polymerase (Sp6, T3, or T7) as suggested by the manufacturer (Stratagene). *In vitro* translation was performed by incubating 1 μg of capped mRNA with rabbit reticulocyte lysate (Promega) and ^{35}S-labeled methionine as described (24). One μl of the reaction mixture was run on a 10% sodium dodecyl sulfate-polyacrylamide gel, dried, and autoradiographed.

Gel Retardation Assay—Protein nuclear extracts were made from human promyelocytic HL-60 cells as described (25). Briefly, 10^7 cells were washed twice in Hank's-buffered saline solution at 4 °C, resuspended in 200 μl of lysis buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 60 mM KCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 5 min. Cellular lysates were then centrifuged, washed in lysis buffer without Nonidet P-40, and the nuclear pellet was resuspended in nuclear resuspension buffer (0.25 M Tris, pH 7.8, 60 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Nuclei were lysed by three cycles of freeze-thawing and nuclear proteins were quantitated by the Bradford method and stored at −80 °C.

Double-stranded oligodeoxynucleotide probes were made using an automated DNA synthesizer. Three probes were made by synthesizing 24-nt oligomers (sense and antisense strands) and combining them in equal molar amounts to form double-stranded fragments. The probes synthesized were:

JUN1CD	5′-GAATGGGAGCGACGACCCGGCCAG-3′ TTACCCTCGCTGCTGGGCCGGTCG
MYBAP1	5′-GAATGGGAGCGATGACTCAGCCAG-3′ TTACCCTCGCTACTGAGTCGGTCG
JUN1CDMUT	5′-GAATGGGAGAAACTTCCCGGCCAG-3′ TTACCCTCTTTGAAGGGCCGGTCG

Gel retardation assays using *in vitro* translated products were performed as described (17). Briefly, 1/20 of the translation products were incubated with 10^5 cpm of probe and run on 5% 0.25 × TBE acrylamide gel, dried, and autoradiographed (Fig. 4). Gel retardation assay using nuclear extracts were also done as described (25). Briefly, 5 μg of nuclear extracts were incubated with 10^5 cpm of probe plus 3 μg of poly(dI-dC) and binding buffer (10 mM Hepes, pH 7.9, 60 mM KCl, 4.0% Ficoll, 1 mM dithiothreitol, and 1 mM EDTA). Binding reactions were run on 5% Tris-glycine (Fig. 3) or 4% 0.25 × TBE (Fig. 5) gels, dried, and audioradiographed.

Assays using JunD antiserum (a kind gift from Dr. R. Bravo, Squibb, Princeton, NJ) were as described (26).

Cell Culture and RNA Phenotyping by Reverse Transcriptase-Polymerase Chain Reaction Analysis—Peripheral blood mononuclear cells (PBMC) isolated from 50 ml of venous blood of healthy donors by Ficoll-Hypaque (Sigma) gradients were comprised of 70% T-lymphocytes, 10% B-lymphocytes, and 20% monocytes as determined by flow cytometry. Monocyte-macrophages were partially removed

[1] The abbreviations used are: CAT, chloramphenicol acetyltransferase; nt, nucleotide(s); bp, base pair(s); PCR, polymerase chain reaction; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

by two-step (1 h each) plastic adherence. Residual nonadherent cells were cultured in 24-well microtiter plates (Costar, Cambridge, MA) in RPMI 1640 medium containing 10% (v/v) heat inactivated fetal bovine serum, antibiotics, and 5 μg of phytohemagglutinin (PHA) (GIBCO) per ml at 37 °C in a humidified (5% CO_2, 95% air) incubator at 2×10^5/ml. Cells were cultured for 0, 2, 4, 8, 16, 24, 36, and 48 h, harvested by centrifugation, washed twice in 0.9% NaCl solution, and total RNA extracted as described (5). junD, c-*myb*, and B_2 microglobulin mRNA levels were measured by reverse transcriptase-PCR with 5′ and 3′ primers specific for each mRNA and detected by hybridization with specific probes. Primers and probes for c-*myb* and β_2-microglobulin mRNAs were as described (7); the c-*jun* 5′ primer was 5′-ACCTTCTATGACGATGCCCTCA-3′ (beginning 22 nucleotides downstream of the initiation codon), the 3′ primer was 5′-CCTGCTCATCTGTCACGTTCTT-3′ (beginning 301 nucleotides downstream of the initiation codon), the hybridization probe was 5′-AGCCGCACCTCCGCGCCAAGAA-3′ (beginning 149 nucleotides downstream from the initiation codon); all were derived from the published sequence of the human c-*jun* cDNA (27). The junD 5′ primer was 5′-TTGTCGCCCATCGACATGGACA-3′ (nt 639–660), the 3′ primer was 5′-AGCTCCGTGTTCTGACTCTTGA-3′ (nt 776–797), the hybridization probe was 5′-TCTTCACTTTCTCT-TCCAGGCG-3′ (nt 751–772); all were derived from the published sequence of the human junD cDNA (28).

Oligomer Treatment of PBMC—PHA-stimulated PBMC, cultured at a density of 1×10^5/ml in 24-well plates (Costar, Cambridge, MA) as described above, were either untreated or exposed to c-*jun* or junD sense or antisense oligodeoxynucleotides (40 μg/ml at 0 time, 20 μg/ml at 24 h) (reviewed in Ref. 29). After 5 days, cells (mean + S.D. of experiment performed in triplicate) were counted. The sequence of the c-*jun* sense oligomer (codons 1–6) was 5′-ATGACTGCAAA-GATGGAA-3′: the sequence of the c-*jun* antisense oligomer (complementary to codons 1–6) was 5′-TTCCATCTTTGCAGTCAT-3′. The sequence of the junD sense oligomer (codons 1–6) was 5′-ATGAA-GAAGGACGCGCTG-3′; the sequence of the junD antisense oligomer (complementary to codons 1–6) was 5′-CAGCGCGTCCTTCTT-CAT-3′.

RESULTS

Transactivation by Jun Family Genes of CAT Gene Expression Driven by the Human c-myb 5′-Flanking Region—The ability of the three Jun family members (c-Jun, JunB, and JunD) to transactivate CAT reporter constructs containing 687 (B1), 197 (S1), or 78 (P1) nucleotides of the human c-*myb* 5′-flanking region (Fig. 1*B*) was assessed in transient expression assays using Syrian hamster fibroblast Tk-ts13 (21) transfected at a 2:1 effector-to-reporter ratio and assayed 48 h later for enzyme activity. The SV40 c-*jun* effector plasmid induced little transactivation of the P1CAT reporter construct but determined a 6–8-fold increase in CAT expression driven by the S1 and B1 promoters (Fig. 2*A*). The expression of JunB had little or no effect on any of the reporter constructs (Fig. 2*B*). The junD effector plasmid induced a 10–15-fold increase in CAT expression in the S1 and B1 CAT reporters but only a marginal increase in activity of the most proximal P1CAT reporter unit (Fig. 2*C*), suggesting the presence of a cis-acting element within the segment from −197 to −78 of the human c-*myb* 5′-flanking region that interacts with c-Jun and JunD proteins.

Sequence analysis revealed an element similar to the consensus Ap1-binding site, with three mismatches, centered at position −149 of the c-*myb* promoter.

Mutations of the Ap1-like Element in the c-myb 5′-Flanking Region Abolishes c-Jun and JunD Transactivation of CAT Expression—To demonstrate that the sequence centered at position nt −149 of the human c-*myb* promoter accounts for c-Jun and JunD protein binding and transactivation, we analyzed the ability of c-Jun and JunD to transactivate a CAT reporter construct containing site-directed mutations in the Ap1-like-binding site derived by PCR amplification of the region −164 to +80 of the human c-*myb* gene followed by linkage to the CAT reporter gene (Fig. 3*A*). The introduction of mutations in the binding site essentially abolished the transactivation of c-Jun and JunD on this segment of the c-

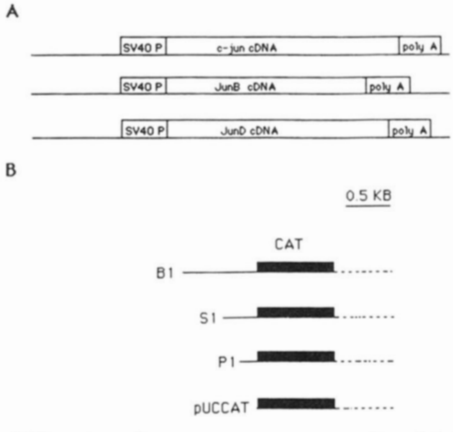

FIG. 1. **Effector and reporter constructs.** *Panel A*, Jun effector plasmids in which murine jun cDNAs are under the control of the SV40 early promoter and polyadenylation signal. *Panel B*, reporter constructs in which different segments of the human c-*myb* promoter drive the bacterial CAT gene. *B1CAT*, CAT gene linked to the *Bam*HI-*Nco*I fragment (from −687 to +204) of the human c-*myb* gene; *S1CAT* consists of the bacterial CAT gene linked to the *Sma*I-*Nco*I fragment (nt −197 to +204) of c-*myb* gene; *P1CAT*, consists of the CAT gene linked to the *Pst*I-*Nco*I fragment (nt −78 to +204) of the c-*myb* gene. ——, human c-*myb* sequences; – – –, pUC9 sequences.

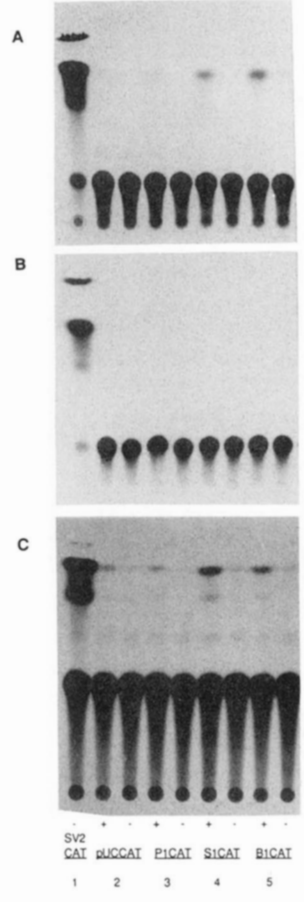

FIG. 2. **Autoradiograms of CAT assays.** CAT activity was measured in lysates of cells transfected with the reporter construct in the presence (+) or absence (−) of the pSVc-jun (*panel A*), pSVjunB (*panel B*) or pSVjunD (*panel C*) effector gene. Reporter constructs are listed at the *bottom* of each *lane*. Results from five separate assays were superimposable.

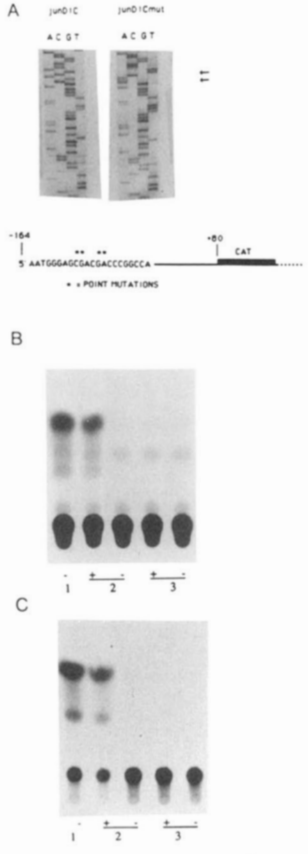

FIG. 3. **CAT assays using a mutated Ap1-like-binding site.** *Panel A*, the mutated sequence resulting from site-directed mutagenesis and the construct diagram. *Panel B*, autoradiogram shows CAT activity on: *lane 1*, cells transfected with pSV2CAT as positive control; *lane 2*, lysates of cells transfected with the wild-type reporter construct (pJUN1CWTCAT) with (+) or without (−) the pSVc-jun effector gene; *lane 3*, cells transfected with the mutant reporter construct (pJUN1CMUTCAT) with (+) or without (−) the pSVc-jun effector gene. *Panel C*, autoradiogram shows CAT activity as in *panel B* except that the effector gene is pSVjunD.

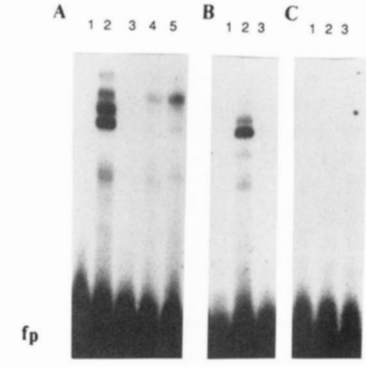

FIG. 4. **Gel retardation assay for Jun-binding sites.** Synthetic oligonucleotides containing the putative Jun binding region were assayed for binding to HL-60 nuclear extracts. Binding reactions were loaded onto a 5% high ionic strength Tris-glycine polyacrylamide gel. *Panel A*, MYBAP1 in which the Ap1-like-binding site centered at position −149 of the human c-*myb* promoter was mutated into a canonical Ap1-binding site. *Lane 1*, probe only; *lane 2*, probe plus HL-60 nuclear extract; *lanes 3–5*, competition assays using the probe JUN1CD which contains the wild-type sequence centered at position lms149 as competitor at 1000-fold (*lane 3*), 500-fold (*lane 4*), or 100-fold (*lane 5*) molar excess of competitor. *Panel B*, JUN1CD probe. *Lane 1*, probe only; *lane 2*, probe plus HL-60 nuclear extract; *lane 3* MYBAP1 as competitor at 1000-fold molar excess. *Panel C*, JUN1CDMUT randomly mutated probe. *Lane 1*, probe only; *lanes 2* and *3*, probe plus 5 and 10 μg of HL-60 nuclear extract respectively; *fp* indicates unbound probe.

myb 5′-flanking sequence (Fig. 3, *B* and *C*, *lane 3*), while a similar construct containing wild-type sequences from nt −164 to +80 of the human c-*myb* gene gave a 5–10-fold increase in CAT activity (Fig. 3, *B* and *C*, *lane 2*). These data further demonstrate that the Ap1-like element at position −162 to −138 is directly involved in c-Jun and JunD transactivation of the human c-*myb* promoter.

Binding of c-Jun and JunD Protein to a Putative Jun-binding Site—A synthetic fragment containing the putative JunD-binding element at position −149 was used as probe to determine whether this sequence binds to nuclear proteins in gel retardation assays. Three 24-bp oligodeoxynucleotides from the region −162 to −138 of the human c-*myb* promoter were synthesized: 1) the wild-type sequence (JUN1CD), 2) an oligonucleotide in which the putative Ap1-like motif was mutated into a canonical Ap1 motif by changing 5′-GAGCGACGACCCGG-3′ to 5′-GAGCGATGACTCAG-3′ (MYBAP1), based on previous reports (32, 45) that all three Jun proteins bind to the Ap1 site, and 3) a randomly mutated oligonucleotide with the sequence 5′-GAGAAACTTCCCGG-3′ (JUN1CDMUT). Gel retardation assays with the 24-bp MYBAP1 fragment revealed DNA-protein complexes (Fig. 4*A*, *lane 2*). Incubation with increasing amounts of JUN1CD oligomers containing the wild-type sequence as competitor (*lanes 5–3*) abolished the three retarded complexes indicating that the element centered at −149 of the c-*myb* 5′-flanking sequence competes for protein binding to canonical Ap1 sites. Likewise, use of MYBAP1 as competitor for JUN1CD binding of nuclear proteins revealed competition for the formation of complexes with a canonical Ap1-binding site (Fig. 4*B*, *lane 3*). The mutant probe JUN1CDMUT formed no detectable DNA-protein complex (Fig. 4*C*).

To determine whether Jun proteins actually bound to the −162 to −138 segment of the c-*myb* promoter, *in vitro*-translated proteins were used in gel retardation assays. Proteins of the correct size were synthesized in reactions utilizing the sense jun mRNAs, whereas no protein was generated in reactions using the antisense mRNAs (data not shown). These *in vitro* synthesized proteins were reacted with the JUN1CD wild-type probe or the mutated MYBAP1 fragment containing a canonical Ap1 site. The JunB protein did not bind to either probe, whereas the c-Jun and JunD proteins bound only to the canonical Ap1 probe (Fig. 5). Ryseck and Bravo (30) reported that flanking regions were important for Jun binding (both canonical and Ap1-like sequences) and showed that of all the Ap1-like sequences reported to bind Jun proteins only a few bound to Jun homodimers. In contrast, Jun proteins could bind to the majority of Ap1-like sequences in the heterodimeric form with one of the fos family members (c-*fos*, FOSB, or Fra-1) (31). Because this might have also occurred in our experiments, we used a JunD-specific antiserum that detects the N terminus of the protein which is upstream of the leucine zipper and DNA-binding domain, and that can immunoprecipitate JunD in complex with Fos proteins (31). Nuclear extracts were incubated first with the JunD-specific antibody and then with the JUN1CD (Fig. 6, *lanes 1–6*) or MYBAP1 oligonucleotide probe (Fig. 6, *lanes 7–12*) and subjected to gel retardation analysis. The lanes in which the antibody was added (*lanes 4–6* and *10–12*) showed the expected "supershifted" complex, reflecting interaction of the antibody with the JunD protein, that was more apparent with increasing amounts of antibody. *Lanes 3* and *9*, in which the nuclear proteins were incubated with rabbit preimmune serum, showed no supershifted complex, demonstrating the

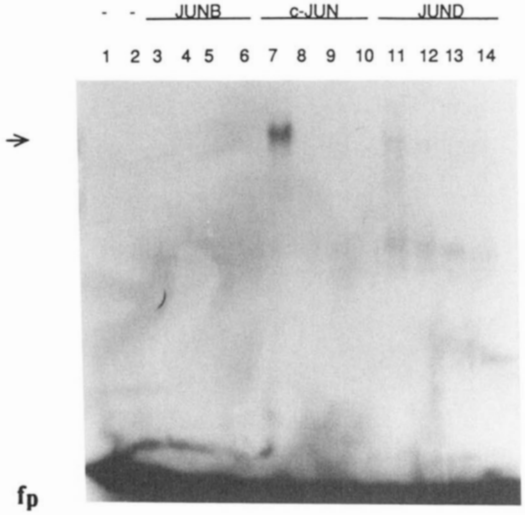

FIG. 5. **Gel retardation assays using *in vitro* translated Jun proteins.** *Lanes 1* and *2*, reticulocyte lysate only; *lanes 3* and *4*, *in vitro* translated sense JunB; *lanes 5* and *6*, *in vitro* translated antisense JunB; *lanes 7* and *8*, *in vitro* translated sense c-Jun; *lanes 9* and *10*, *in vitro* translated antisense c-Jun; *lanes 11* and *12*, *in vitro* translated sense JunD; *lanes 13* and *14*, *in vitro* translated antisense JunD. In *odd-numbered lanes*, MYBAP1 was used as probe; in *even-numbered lanes* JUN1CD was used as probe. *Arrow* indicates the specific DNA-protein complex; *fp* indicates unbound probe.

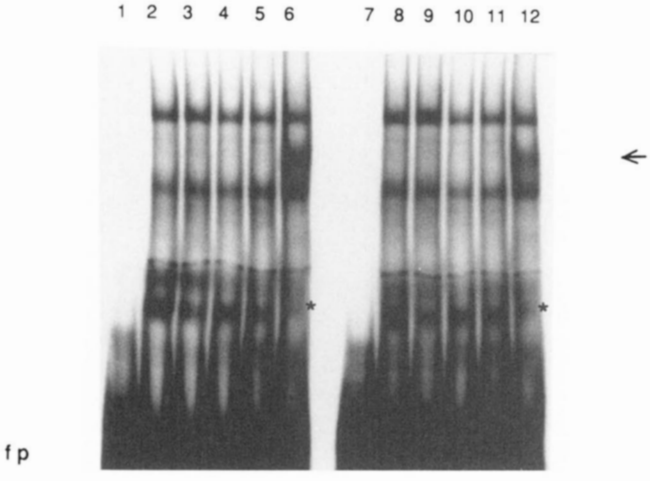

FIG. 6. **Gel retardation assays with HL-60 nuclear extract in the presence of a JunD-specific antiserum.** Nuclear extracts were first incubated with a JunD-specific antiserum and then with an oligoprobe containing the Ap1-like element at −149 of the human c-*myb* promoter. Binding reactions were loaded onto a 4% low ionic strength 0.25 × TBE polyacrylamide gel. *Lanes 1* and *7*, probe only; *lanes 2* and *10*, probe plus nuclear extract; *lanes 3* and *9*, nuclear extracts incubated with 50 μg of preimmune serum; *lanes 4–6* and *10–12*, nuclear extracts incubated with 0.5, 5, or 50 μg of JunD antiserum. *Lanes 1–6*, MYBAP1 probe; *lanes 7–12*, JUN1CD probe. The *arrows* indicate supershifted complexes due to the antibody interaction; the *asterisk* indicates abolished DNA-protein complex due to the supershift; *fp* indicates unbound probe.

specificity of the interaction of JunD protein with the −162 to −138 segment of the c-*myb* promoter probably in conjunction with the protein product of a fos family gene. No supershifted complex was detected when nuclear extracts were incubated with a JunB antiserum (not shown). Because a c-Jun antiserum was not available, a c-*fos* full-length mRNA was cotranslated with c-*jun*, and the translation complex was able to react in gel retardation assays with the −162 to −138 segment (JUN1CD probe) of the c-*myb* promoter (not shown). In contrast, no binding was detected when gel retardation assays were performed with cotranslation products of different combinations of jun cDNAs (not shown).

c-Jun and JunD Cooperate with c-myb in Transactivating the Human c-myb Promoter—The interaction of c-Jun and/or JunD with the regulatory region of the human c-*myb* promoter could be significant because it determines up-regulation of c-*myb* expression which in turn allows for the positive autoregulation or because there is authentic cooperation between c-Jun and/or JunD with Myb in the regulation of c-*myb* expression.

As the first step in investigating the possibility that c-Jun and/or JunD would cooperate with Myb in transactivating the c-*myb* promoter, we measured CAT activity driven by 687 nucleotides (B1 construct) of the human c-*myb* 5′-flanking region in TK-ts13 cells transfected at 2:1 effector-to-reporter ratio with either pSV-c-*jun* or pSV-JunD or pSV-c-*myb* alone or a combination of pSV-c-*jun* and pSV-c-*myb* or pSV-junD and pSV-c-*myb*. The cotransfection of junD and c-*myb* effector plasmids (Fig. 7, *lane 4*) or c-*jun* and c-*myb* effector plasmids (Fig. 7, *lane 8*) resulted in higher levels of CAT expression than by transfection of equal amounts of either junD or c-*jun* or c-*myb* alone (Fig. 7, *lanes 2*, *3*, *6*, and *7*) suggesting that c-Jun and/or JunD might cooperate with Myb in the transcriptional regulation of c-*myb* expression.

Expression of c-jun and JunD mRNA in PHA-stimulated PBMC—c-*jun* and JunD transactivation of reporter gene expression driven by the c-*myb* 5′-flanking sequence could be functionally relevant in cells with detectable levels of c-*myb* mRNA. Because c-*myb* expression is induced in late G_1 by PHA treatment of PBMC, we used this system to determine whether c-*jun* and junD mRNAs are detected at an earlier time than c-*myb* mRNA. c-*jun* mRNA was first detected after 2 h and remained essentially constant throughout the entire time course; junD mRNA was first detected after 8 h of culture, reaching a peak level at 16 h and remaining at similar levels throughout the time course whereas c-*myb* mRNA was

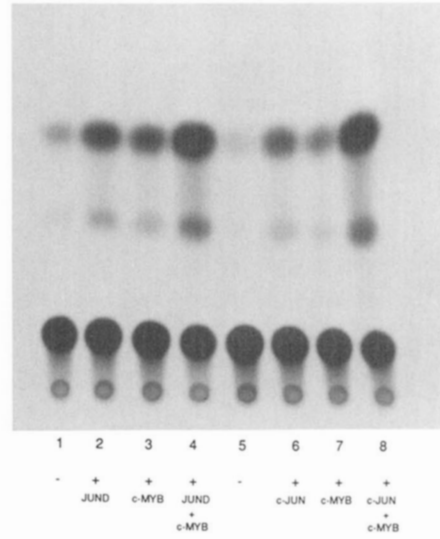

FIG. 7. **c-jun and JunD cooperate with c-*myb* in transactivating the human c-*myb* promoter.** Tk-ts13 cells were transfected with the B1CAT reporter plasmid with or without pSVc-jun, pSVjunD, or pSV40 c-*myb* effector plasmids. *Lanes 1* and *5*, cells transfected with the B1CAT reporter plasmid only; *lane 2*, cells cotransfected with the B1CAT reporter plus the pSVjunD effector plasmid; *lanes 3* and *7*, cells cotransfected with B1CAT reporter plasmid plus pSV40 c-*myb* effector plasmid; *lane 4*, cells cotransfected with B1CAT reporter plasmid plus pSVjunD and pSV40c-*myb* effector plasmid; *lane 6*, cells cotransfected with B1CAT reporter plasmid plus pSVc-jun effector plasmid; *lane 8*, cells cotransfected with B1CAT reporter plus pSVc-jun and pSV40c-*myb* effector plasmids.

sharply increased over basal levels at 24 h and expression levels remained constant up to 48 h (Fig. 8). Expression of β_2-microglobulin mRNA (used as control) was constant throughout the culture period.

Effect of c-jun and JunD Antisense Oligodeoxynucleotides on T-lymphocyte Proliferation—To determine whether down-regulation of c-*jun* and junD expression affects T-lymphocyte proliferation, PBMC which normally reside in the G_0 phase of the cell cycle, were stimulated to proliferate by PHA treatment in the presence or absence of c-*jun* or junD oligodeoxynucleotides. After 5 days in culture, cells were enumerated. Cell numbers (mean ± SD) in the PHA-treated control and c-*jun* sense-treated cultures were similar ($5.3 \times 10^5 \pm 5 \times 10^4$ *versus* $4.3 \times 10^5 \pm 3.0 \times 10^4$) whereas T-lymphocyte proliferation was inhibited (46%) in the c-*jun* antisense-treated cell suspensions ($2.6 \times 10^5 \pm 3.2 \times 10^4$) as compared to the sense-treated cultures (Fig. 9*A*). Cell numbers (mean ± S.D.) in the PHA-treated control and junD sense-treated cultures were similar ($4.6 \times 10^5 \pm 2.0 \times 10^4$/ml *versus* $4.2 \times 10^5 \pm 3.5 \times 10^4$/ml), whereas T-lymphocyte proliferation was inhibited (43%) in the antisense-treated cell suspensions ($2.6 \times 10^5 \pm 1.5 \times 10^4$/ml) as compared to the sense-treated cultures (Fig. 9*B*). To determine whether this inhibition correlated with c-*jun* and junD transcript levels, total RNA was extracted from each sample 24 h after exposure to 40 μg/ml of c-*jun* or junD oligodeoxynucleotides and c-*jun* or junD expression was measured by reverse transcriptase-PCR; c-*jun* or junD mRNA was barely detectable in antisense-treated cells, but abundantly expressed in sense-treated and untreated cells (not shown). Moreover, treatment of PBMC with c-*jun* or junD antisense oligodeoxynucleotides was associated with down-regulation of c-*myb* expression as indicated by the decrease of c-*myb* mRNA levels in antisense-*versus* sense-treated cultures (Fig. 10).

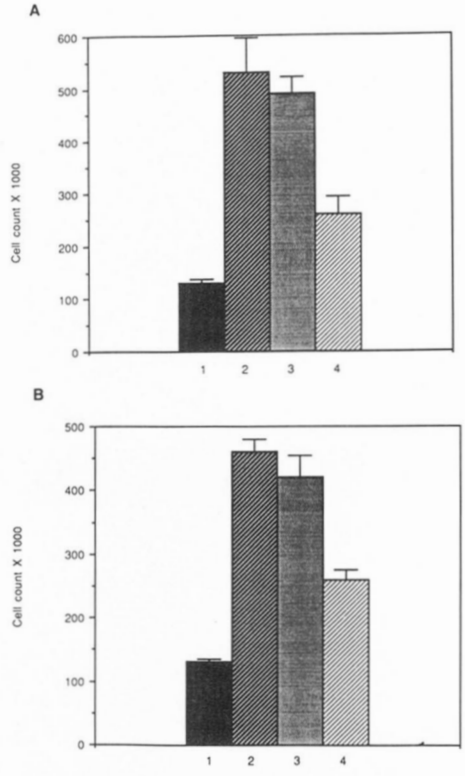

FIG. 9. **Effect of c-*jun* or JunD antisense oligodeoxynucleotides on PHA-stimulated T-lymphocyte proliferation.** PBMC were stimulated to proliferate by culturing in the presence of PHA only or exposed to PHA and c-*jun* (*panel A*) or junD (*panel B*) sense or antisense oligodeoxynucleotides. After 5 days, cell counts (mean ± S.D. of experiment performed in triplicate) were performed. *Lane 1*, untreated PBMC; *lane 2*, PHA-stimulated PBMC; *lane 3*, PHA-stimulated PBMC exposed to sense oligodeoxynucleotides; *lane 4*, PHA-stimulated PBMC exposed to antisense oligodeoxynucleotides.

DISCUSSION

The protooncogene c-*myb* appears to play an essential role in the regulation of hematopoietic cell proliferation because inhibition of Myb protein synthesis prevents G_1/S transition in those cells (5–7, 32–34); however, the molecular mechanisms governing the expression of c-*myb* in that phase of the cell cycle are still poorly understood. To study the transcriptional regulation of c-*myb* expression, we previously isolated a genomic clone containing the 5′-flanking region of the human c-*myb* gene (17). We identified multiple Myb-binding sites from nucleotide −575 to −616 from the major cap site of the human c-*myb* gene; upon binding to Myb protein, these elements activate the expression of reporter constructs regardless of the orientation, which is characteristic of a "typical" enhancer (35). Furthermore, these Myb-binding sites appear to be involved in the cell type-specific pattern of c-*myb* expression since promoters containing these binding sites determine a high level of reporter gene expression in cell lines of hematopoietic origin, but are silent in fibroblasts.[2]

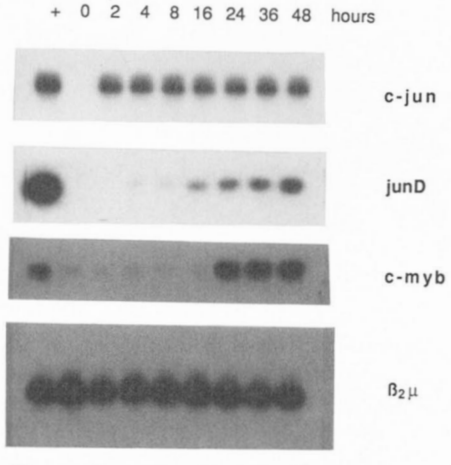

FIG. 8. **Expression of c-*jun*, JunD, c-*myb*, and β_2-microglobulin (β-2) mRNAs in PHA-stimulated PBMC.** PBMC were stimulated with PHA (5 μg/ml) as described under "Materials and Methods"; total RNA was isolated at different times of culture, reverse-transcribed, and amplified with primers specific for each mRNA; mRNA expression was detected by hybridization with synthetic ^{32}P-end-labeled oligomers specific for each amplified product. (+) *lane* is HL-60 RNA amplified as positive control.

We hypothesize that the Myb-binding sites mediate positive autoregulation of c-*myb* to prolong or amplify its expression at the G_1/S boundary where Myb protein appears to exert its primary function. We undertook this investigation to determine whether proteins expressed in early G_1 might be involved in the activation of c-*myb* expression up to a level required for the functioning of the autoregulatory mechanism involving Myb protein itself. Genes of the jun family appeared likely candidates for such a role because their expression is rapidly induced in cells stimulated by growth factors or mitogens (36–40). In addition, these genes appear to function in the nucleus due to the interaction of the encoded proteins with specific DNA-binding sites (41, 42) in a dimeric form through a leucine zipper motif which allows the interaction of a Jun protein with itself or with proteins of the fos gene family (24, 30, 43). Our results show that c-Jun and JunD strongly transactivate

[2] R. Gualdi, manuscript submitted.

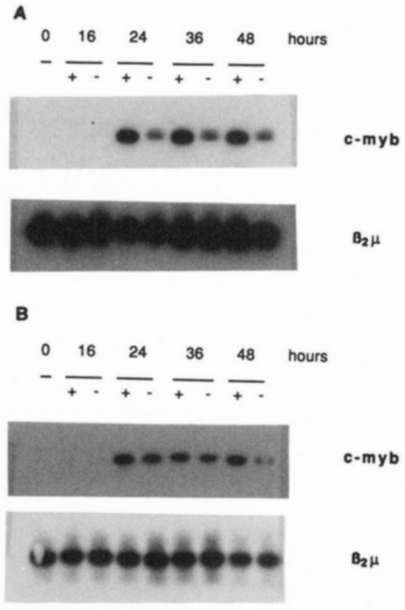

FIG. 10. **Expression of c-*myb* mRNA in PHA-stimulated PBMC exposed to c-*jun* or JunD antisense oligodeoxynucleotides.** Total RNA was isolated from PBMC and reverse-transcribed, amplified with c-*myb*-specific primers and hybridized to a synthetic 50-base c-*myb* fragment localized in the amplified c-*myb* cDNA sequence (nt 2, 351–2, 400) as described (7). *Numbers* indicate the times (h) at which PBMC were collected and RNA was extracted after PHA stimulation. Exposure to c-*jun* or junD sense (*lanes* +) or antisense (*lanes* −) oligodeoxynucleotides was as described under "Materials and Methods." *Panel A*, PBMC exposed to c-*jun* oligodeoxynucleotides; *panel B*, PBMC exposed to junD oligodeoxynucleotide.

the expression of a reporter gene linked to the c-*myb* promoter, whereas JunB has no effect, via an element that appears to bind c-Jun and JunD as a heterodimer probably with a member of the fos family since Jun/Fos complexes are known to bind effectively to Ap1-like sequences (30). We did not observe binding of the JunB, c-Jun, or JunD proteins to the wild-type Ap1-like probe in gel retardation assays with *in vitro* translated proteins probably because binding to Ap1-like sequences can only occur with heterodimers (30).

Studies of c-Jun or JunD binding performed with canonical Ap1 sequences clearly demonstrate that the proteins have affinity for the site, as a homodimer and/or as a heterodimer depending on the 5′- and 3′-flanking region (30). However, most Ap1-like-binding sites are unable to bind c-Jun or JunD as homodimer, but can bind as a heterodimer, consistent with our data. It remains to be determined whether the higher transactivating activity of JunD protein is due to a higher binding affinity as compared to the other Jun family members for the segment containing the Ap1-like element. This does not seem unreasonable in light of the ability of other DNA-binding transactivator proteins such as c-*myb* and B-Myb to bind to the same set of sequences and yet recognize distinct high affinity binding sites (44).

One possible consequence of the interaction of c-Jun and/or JunD protein with the 5′-flanking region of the human c-*myb* promotor is to increase c-*myb* basal levels, either by itself or in cooperation with other transacting factors, within the promoter and/or introns. This level of c-*myb* expression would then allow Myb to bind to its own promoter and maintain enhanced transcription during G1/S transition or in S phase.

This hypothesis is consistent with the findings that c-*jun* and junD expression precedes that of c-*myb* in mitogen-stimulated human T-lymphocytes (Fig. 8) and that treatment of these cells with c-*jun* and junD antisense oligodeoxynucleotides inhibits proliferation (Fig. 9). The finding that c-*jun* or junD antisense oligomers inhibited T-cell proliferation by only 46 and 43%, respectively, might be explained due to the ability of the remaining Jun protein to direct cellular proliferation. In fact, experiments in which T-cells were treated with both c-*jun* and junD antisense oligos resulted in even a greater inhibition on cellular proliferation (~65%) (not shown). The findings that microinjection of c-Jun or JunD antibodies into Swiss 3T3 fibroblasts considerably inhibits (~50%) DNA synthesis in those cells (45) are also compatible with the hypothesis that c-Jun and JunD act by regulating the expression of other genes in the late G_1 phase of the cell cycle. Although c-*myb* is not expressed in fibroblasts, c-Jun or JunD might be functionally linked to a c-*myb* equivalent such as B-myb (46) whose expression also increases at the G_1/S transition (46) and appears to be required for fibroblast proliferation.[3] Finally, introduction of a constitutively expressed c-*jun* or junD into murine erythroleukemia cells exerts an effect similar to that caused by c-*myb* constitutive expression (49–51) because it blocks the ability of those cells to differentiate into erythroblasts in the presence of dimethyl sulfoxide (52). Although c-Jun, JunD, and Myb might act through entirely independent pathways, the identification of a direct interaction of c-Jun and JunD proteins with the c-*myb* 5′-flanking region raises the possibility that these genes are functionally linked to allow amplification of the mitogenic signal transmitted by Myb. The observation that the cotransfection of c-Jun or JunD with Myb determines higher levels of CAT activity than either c-Jun, JunD, or Myb alone does supports this possibility. The demonstration that Myb interacts synergistically with the Epstein-Barr virus BZLF1 transactivator in lymphoid cells (48) is also consistent with the possibility that Myb cooperates with other transactivators.

The generation of hybrid constructs containing individual or double mutations of the Ap1-like and myb-binding sites and their introduction into a fibroblast cell line constitutively expressing c-*myb* will allow more detailed analysis of the relative contribution of JUN and c-*myb* in the regulation of c-*myb* expression. These experiments are now in progress. If the role of c-Jun and/or JunD is simply to increase c-*myb* basal levels and thereby activate the positive autoregulation of c-*myb* expression Jun$^-$ myb$^+$ and Jun+ myb+ constructs would be transactivated by a constitutively expressed c-*myb* with equal efficiency. However, if Jun proteins cooperate with Myb in the regulation of c-*myb* expression, the Jun$^-$ myb$^+$ construct would be transactivated less efficiently than the Jun$^+$ myb$^+$ construct in fibroblast cells with endogenous Jun levels and constitutive expression of c-*myb*.

A better definition of the interrelationship between Jun genes and c-*myb* awaits further experimental analysis.

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REFERENCES

1. Moscovici, C. (1975) *Curr. Top Microbiol. Immunol.* **71,** 79–96
2. Moscovici, C., and Gazzolo, L. (1982) in *Advances in Viral Oncology*, Vol. 1, pp. 83–106, Raven Press, New York
3. Westin, E. H., Gallo, R. C., Arya, S. K., Eva, A., Souza, L. M., Baluda, M. A., Aaronson, S. A. and Wong-Staal, F. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2194–2198
4. Duprey, S. P., and Boettinger, D. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 6937–6941
5. Gewirtz, A. M., Anfossi, G., Venturelli, D., Valpreda, S., Sims, R., and Calabretta, B. (1989) *Science* **245,** 180–183
6. Furukawa, Y., Piwnica-Worms, H., Ernst, T. J., Kanakura, J., and Griffin,

[3] A. Sala and B. Calabretta, submitted for publication.

J. D. (1990) *Science* **250,** 805–808
7. Venturelli, D., Travali, S., and Calabretta, B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5963–5967
8. Travali, S., Reiss K., Ferber, A., Mercer, W. E., Petralia, S., Calabretta, B., and Baserga, R. (1991) *Mol. Cell. Biol.* **11,** 731–736
9. Klempnauer, K. H., Symonds, G., Evan, G. I., and Bishop, J. M. (1984) *Cell* **37,** 537–547
10. Boyle, W. J., Lampert, M. A. Lipsick, J. S., and Baluda, M. A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 4265–4269
11. Moelling, K., Pfoff, E., Beug, H., Beimling, P., Bunte, T., Schaller, H. E., and Graf, T. (1985) *Cell* **40,** 983–990
12. Klempnauer, K. H., and Sippel, A. E. (1987) *EMBO J.* **6,** 2719–2725
13. 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
14. Biedenkapp, H., Borgmeyer, U., Sippel, A. E., and Klempnauer, K. H. (1988) *Nature* **335,** 835–837
15. Nakagoshi, H., Nagase, T., Kanei-Ishii, C., Ueno, Y., and Ishii, S. (1990) *J. Biol. Chem.* **265,** 3479–3483
16. Dasgupta, P., Saikumar, P., Reddy, C. D., and Reddy, E. P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8090–8094
17. Nicolaides, N. C., Gualdi, R., Casadevall, C., Manzella, L., and Calabretta, B. (1991) *Mol. Cell. Biol.* **11,** 6166–6176
18. Ness, S. A., Marknell, A., and Graf, T. (1989) *Cell* **59,** 1115–1125
19. Weston, K., and Bishop, J. M. (1989) *Cell* **58,** 85–93
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY
21. Talavera, A., and Basilico, C. J. (1977) *Cell. Physiol.* **92,** 425–436
22. Huttner, K. M., Barbosa, J. A., Scangos, G. A., Pratcheva, D. D., and Ruddle, F. H. J. (1981) *Cell. Biol.* **91,** 153–156
23. Kreig, P., and Melton, D. (1987) *Methods Enzymol.* **155,** 397–426
24. Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E., and Leder D. (1988) *Cell* **55,** 917–924
25. Stein, B., Rahmsdorf, H. J., Steffen, A., Liftin, M., and Herrlich, P. (1989) *Mol. Cell. Biol.* **9,** 5169–5181
26. Ogas, J., Andrews, B. J., and Herskowitz, I. (1991) *Cell* **66,** 1015–1026
27. Hattori, K., Angel, P., LeBeau, M. M. and Karin, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 9148–9152
28. Nomura, N., Ide, M., Sasamoto, X., Matsui, M., Date, T., and Ishizaki, R. (1990) *Nucleic Acids Res.* **18,** 347–348
29. Calabretta, B. (1991) *Cancer Res.* **51,** 4505–4510
30. Ryseck, R. P. and Bravo, R. (1988) *Oncogene* **6,** 533–542
31. Kovary, K., and Bravo, R. (1991) *Mol. Cell. Biol.* **11,** 2451–2459
32. Gewirtz, A. M., and Calabretta, B. (1988) *Science* **242,** 1303–1306
33. Anfossi, G., Gewirtz, A. M., and Calabretta, B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3379–3383
34. Caracciolo, D., Venturelli, D., Valtieri, M., Peschle, C., Gewirtz, A. M., and Calabretta, B. (1990) *J. Clin. Invest.* **85,** 55–61
35. Muller, H. P., and Schaffer, W. (1990) *Trends Genet.* **6,** 300–304
36. Lamph, W. W., Wamsley, P., Sassone-Corsi, P., and Verma, I. M. (1988) *Nature* **334,** 629–631
37. Ryder, K., Lau, L. F., and Nathans, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1487–1491
38. Ryder, K., Lanahan, A., Perez-Albuerne, E., and Nathans, D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1500–1503
39. Ryder, K., and Nathans, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8464–8467
40. Ryseck, R. P., Hirai, S. I., Yaniv, M., and Bravo, R. (1988) *Nature* **334,** 535–537
41. Angel, P., Allegretto E., Okino, S., Hattori, K., Boyle, W., Hunter, T., and Karin, M. (1988) *Nature* **332,** 166–171
42. Bohmann, D., Bos, T., Admon, A., Nishimura, T., Vogt, P., and Tjian, R. (1987) *Science* **238,** 1386–1392
43. Nakageppu, Y., Ryder, K., and Nathans, D. (1988) *Cell* **55,** 907–915
44. Mizuguchi, G., Nakagoshi, H., Nagase, T., Nomura, N., Data, T., Ueno, Y., and Ishii, S. (1990) *J. Biol. Chem.* **265,** 9280–9284
45. Kovary, K., and Bravo, R. (1991) *Mol. Cell. Biol.* **11,** 4466–4472
46. Nomura, N. Takahashi, M., Matsui, M., Ishii, S. Date, T., Sasamato, S., and Ishizaki, R. (1988) *Nucleic Acids Res.* **23,** 11075–11084
47. Reiss, K., Travali, S., Calabretta, B., and Baserga, R. (1991) *J. Cell. Physiol.* **148,** 338–343
48. Kenney, S. C., Holley-Guthrie, E., Quinlivan, E. B., Gutsch, D., Zhang, Q., Bender, T., Giot, J. F., and Sergeant, A. (1992) *Mol. Cell. Biol.* **12,** 136–146
49. Clarke, M. F., Kukowska-Latallo, J. F., Westin, E. H., Smith, M., and Prochownick, E. (1988) *Mol. Cell. Biol.* **8,** 884–892
50. McClinton, D., Stafford, J., Brents, D., Bender, T. P., and Kuehl, W. M. (1990) *Mol. Cell. Biol.* **10,** 705–710
51. McMahon, J., Howe, K. M., and Watson, R. J. (1988) *Oncogene* **3,** 717–720
52. Prochownik, E. V., Smith, M., Snyder, K., and Emeagwali, D. (1990) *Blood* **76,** 1830–1837