Yin-Yang 1 Activates the *c-myc* Promoter

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Received 15 June 1993/Returned for modification 20 July 1993/Accepted 31 August 1993

Previous studies on the murine *c-myc* promoter demonstrated that a ubiquitously present protein, common factor 1 (CF1), bound at two sites located -260 and -390 bp from the P1 transcription start site. CF1 has been purified to near homogeneity and shown to be identical to the zinc finger protein Yin-yang 1 (YY1) as judged by similarity of molecular weight and other biochemical properties, immunological cross-reactivity, and the ability of recombinant YY1 to bind to CF1 sites. In cotransfection experiments, YY1 is a strong activator of transcription from *c-myc* promoter-based reporters. Furthermore, in murine erythroleukemia cells, overexpressed YY1 causes increased levels of *c-myc* mRNA initiated from both major transcription initiation sites of the endogenous *c-myc* gene.

Yin-yang 1 (YY1) is a zinc finger protein cloned by Shi et al. (36) in the course of studies on E1A activation of the adeno-associated virus (AAV) P5 promoter. Recombinant YY1 binds a negative regulatory site at -60 and an initiator site at +1 in the AAV P5 promoter. Cotransfected YY1 functions as a repressor of the AAV P5 promoter, and addition of adenovirus E1A protein relieves YY1-dependent repression. Three other groups also cloned cDNAs encoding the YY1 protein by virtue of its ability to bind functionally important sites in unrelated genes, including the immunoglobulin kappa 3' enhancer and the μ E1 site in the immunoglobulin heavy chain (IgH) enhancer (28), the delta sites of ribosomal proteins L30 and L32 (13), and the long terminal repeat of Moloney murine leukemia virus (8). YY1 has subsequently been shown to compete with serum response factor (SRF) for binding to the *c-fos* and skeletal α -actin promoters (10, 21). In the Moloney murine leukemia virus long terminal repeat and the 3' kappa enhancer, the YY1 binding sites are negative sites for transcription (8, 28). Conversely, the IgH μ E1 site (24, 29, 39) and the ribosomal protein delta sites (12, 13) are activator sites, suggesting that YY1 functions as an activator in some gene contexts. Seto et al. (34) also showed that YY1 bound at the +1 site in the AAV P5 promoter functions as a transcriptional initiator. Thus, YY1 is an important regulatory protein with the potential for diverse effects on transcription.

We have previously described a widely expressed DNA-binding protein, CF1, which binds two sites (-390 and -260 bp) in the murine *c-myc* promoter region. On the basis of cross-competition for binding and partial proteolysis, CF1 was also shown to bind the μ E1 site of the IgH enhancer and the downstream CBAR site of the skeletal α -actin promoter (31). On the basis of the results of Park and Atchison showing that recombinant YY1 bound the IgH μ E1 site (28), we suspected that YY1 might correspond to the protein we had identified as CF1. In addition, Atchison et al. identified

a third YY1 binding site in the first *c-myc* exon by virtue of its ability to compete with proteins binding to rpL32 delta sites (1). Thus, we wished to determine the relationship of CF1 to YY1 and to determine how YY1 might affect *c-myc* transcription.

We demonstrate in this paper that YY1 appears to be identical to previously identified CF1, as judged by similarity of mobility in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), binding site specificity, and immunological cross-reactivity. Furthermore, we show that, in a cotransfection assay, recombinant YY1 is a strong activator of a reporter construct dependent on the murine *c-myc* promoter. The *c-myc* promoter is the first example of a natural promoter which is activated by cotransfected YY1. Finally, we show that overexpression of exogenous YY1 causes increased mRNAs initiating from both P1 and P2 promoters of the endogenous *c-myc* gene. These results demonstrate that YY1 binds in the *c-myc* promoter and activates *c-myc* transcription.

MATERIALS AND METHODS

Plasmids and molecular cloning. pGEM-hYY1 contains the human YY1 (hYY1) coding sequence cloned at the *EcoRI* site downstream of the T7 promoter in pGEM7zf(+) (Promega). To create pCMV-hYY1, the cDNA fragment was excised by *ApaI*-*ClaI* digestion, end filled, and cloned into the end-filled *Bam*HI site of the pCMV eukaryotic expression vector (18). pCMV-hYY1DZnF lacks 83 amino acids from the C-terminal end of the protein and was made by blunt-end cloning of the *ApaI*-*HindIII* fragment of pGEM-hYY1 into the *Bam*HI site of pCMV. The plasmids expressing the 12S or 13S E1A gene products, pCMV-12S-E1A and pCMV-13S-E1A, were kindly provided by E. White and have been previously described (41). The pBBLuc, pSNLuc, and pANLuc constructs were made by isolating the indicated fragments of the murine *c-myc* promoter (BB, *Bgl*III [-1139] to *Bgl*III [+571]; SN, *Sma*I [-424] to *Not*I [+334]; and AN, *Ava*I [-139] to *Not*I [+334]), end filling, and inserting the fragment into the *Sma*I site of the p19Luc plasmid (40). The pmmSNLuc plasmid was made by making

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oligonucleotide-directed mutations at the -260 and -390 YY1 sites in the pSNLuc construct. The nucleotides from -252 to -268 were changed to GAATTC, and the nucleotides from -390 to -395 were changed to AATATT. The mutations were shown by electrophoretic mobility shift assay (EMSA) to be unable to bind purified YY1 (data not shown). All nucleotide numbers are relative to promoter P1. The pBBLucDN:X plasmid was created by excising the sequences between the *NotI* site at +335 and the *XhoI* site at +516 nucleotides from pBBLuc and subjecting them to end filling and religation. pBBCAT was previously described (17). pSV2Luc was made by excising the chloramphenicol acetyltransferase (CAT) gene from pSV2CAT (11) with *HindIII* and *BamHI*, end filling, and insertion of the end-filled *BamHI* fragment from p19Luc carrying the luciferase coding sequences.

Protein purification. Nuclear proteins were isolated from the livers of Sprague-Dawley rats, by a modification of the protocol of Dignam et al. (5). Briefly, rat livers were homogenized in $1/2 \times$ TKM buffer containing 25 mM Tris-HCl (pH 7.5), 25 mM KCl, and 7.5 mM $MgCl_2$ and washed once in this buffer. Cells were disrupted by addition of disruption buffer containing 25 mM Tris-HCl (pH 7.5), 25 mM KCl, 7.5 mM $MgCl_2$, 30% sucrose, and 0.5% Nonidet P-40 (NP-40). Nuclei were washed twice in reticulocyte standard buffer containing 100 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 5 mM $MgCl_2$ and lysed by addition of 2 nucleus volumes of elution buffer containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 2 mM $MgCl_2$, 400 mM NaCl, and 20% glycerol. Additional NaCl was added to bring the final NaCl concentration to 400 mM. Protein was extracted at 4°C for 30 min and then centrifuged at $100,000 \times g$ for 1 h. The supernatant was passed over a DEAE column, frozen in liquid nitrogen, and stored at -80°C. To obtain purified CF1, proteins from liver nuclei were heated at 68°C for 10 min and centrifuged at $10,000 \times g$ for 15 min. The supernatant was dialyzed into CF1 buffer (50 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 6 mM $MgCl_2$, 20% glycerol) containing 100 mM NaCl. Proteins were separated by fast protein liquid chromatography (FPLC) chromatography on a Mono Q column (Pharmacia). Bound proteins were eluted with a linear gradient of 0.1 to 0.5 M NaCl in CF1 buffer. Fractions containing CF1 were identified by EMSA analysis, pooled, and dialyzed into CF1 buffer containing 10 mM NaCl. Following dialysis, NP-40 was added to a final concentration of 0.01%, and 700 ng of sheared poly(dI-dC) · poly(dI-dC) (Pharmacia) per μg of total protein was added. The protein was allowed to prebind poly(dI-dC) · poly(dI-dC) for 20 min at 4°C. An oligonucleotide affinity column comprising multimerized copies of a $\mu E1$ site oligonucleotide (31) was equilibrated in CF1 buffer containing 10 mM NaCl, 0.1 mg of insulin per ml, and 0.01% NP-40, and protein was loaded onto the column at a flow rate of 0.15 ml/min. The column was washed with CF1 buffer containing 10 mM NaCl, 0.1 mg of insulin per ml, and 0.01% NP-40, and protein was eluted by addition of CF1 buffer containing 1 M NaCl, 0.1 mg of insulin per ml, and 0.01% NP-40. Protein quantitation prior to oligonucleotide affinity purification was performed by the method of Bradford et al. (3). The proteins present following oligonucleotide affinity column purification were detected by silver staining or by staining with Coomassie blue following electrophoretic separation in an SDS-10% polyacrylamide gel.

EMSA. The probe for EMSA analysis extends from the *AvaI* site at -424 nucleotides in the *c-myc* promoter to the *HpaII* site at -211 nucleotides and comprises both the -260 and the -390 CF1 binding sites. The sequences of the $\mu E1$ and $\mu E3$ site

oligonucleotides are as previously described (31). An oligonucleotide comprising the P5+1 YY1 site of AAV was generously provided by Tom Shenk and comprises the sequence 5'-AGG GTCTCCATTTTGAAGCGGG-3'. EMSA reactions contained 50 mM Tris-HCl (pH 7.5), 10% glycerol, probe, competitor as appropriate, and poly(dI-dC) · poly(dI-dC) at 500 ng of poly(dI-dC) per μg of protein (until after the oligonucleotide affinity column purification step, when it was no longer needed). EMSA reactions were electrophoresed in a 6% nondenaturing polyacrylamide gel with $1 \times$ TBE (89 mM Tris-HCl, 849 mM boric acid, 2 mM EDTA) as buffer.

Protein renaturation. Renaturation was as previously described (36).

Complex ablation. Anti-YY1 monoclonal antibodies were obtained (35a), and their characterization will be described in detail elsewhere. Heterologous monoclonal antibodies were obtained from the laboratories of B. Pernis and E. Kabat and recognize unrelated haptens. Antibodies and probe were first added to the EMSA reaction mixture, and then purified CF1 was added. The reaction mixtures were allowed to bind for 20 min before electrophoresis on a 6% nondenaturing polyacrylamide gel.

In Vitro production of hYY1. mRNA was transcribed from the pGEM-hYY1 plasmid by T7 polymerase (New England Biolabs) according to the manufacturer's instructions. hYY1 was produced by in vitro translation in rabbit reticulocyte lysate as outlined in the manufacturer's (Promega) instructions, except that $ZnCl_2$ was added to the reaction mixture at a final concentration of 0.1 mM.

Cell culture and electroporation. NIH 3T3 fibroblasts, Swiss 3T3 fibroblasts, and P3X63-Ag8 plasmacytomas were grown in Dulbecco's modified Eagle medium (GIBCO) containing 10% heat-inactivated fetal calf serum and 20 μg of gentamicin per ml. 1881 pre-B cells, EL4 T cells, and M12 mature B cells were grown in RPMI (GIBCO) containing 10% heat-inactivated fetal calf serum, 50 mM β -mercaptoethanol, and 20 μg of gentamicin per ml. Cells in suspension were maintained at a concentration of less than 1×10^6 cells per ml and were harvested for electroporation at no greater than 60×10^4 cells per ml. Attached cells were passed at confluence and were harvested for electroporation before confluence at no greater than 300×10^4 cells per 10-cm-diameter plate. Between 200×10^4 and 400×10^4 cells were used per individual electroporation. Swiss 3T3 cells were electroporated in 0.2 ml of fresh growth media at 240 V. All electroporations were done at a capacitance of 950 mF. Swiss 3T3 cells were harvested 20 to 22 h after electroporation, while all other cells were harvested 12 to 14 h after electroporation. Cells were lysed in 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), and 1% Triton X-100. Luciferase activity was assayed in a Berthold luminometer, and the light units detected were standardized to light units detected (luciferase units) per 100 μl of volume. For CAT assays, cells were harvested after 48 h and assayed by using standard conditions.

Stable transfection. Stable transfectants of MEL cells were made by electroporating 200×10^4 to 400×10^4 cells in 0.2 ml of fresh growth media at 240 V and 960 mF with 30 μg of pCMV-neo or pCMV-hYY1-neo plasmid DNA. Colonies arising after 2 to 3 weeks of G418 selection (2 mg/ml) were pooled in groups of 50 or more clones.

Riboprobe analysis. Antisense *c-myc* RNA was produced from a plasmid containing a murine genomic *c-myc* fragment extending from the *BglIII* site at -1150 nucleotides (from P1) to the *XhoI* site at +516 nucleotides cloned into the *BamHI*

TABLE 1. Purification of CF1

Step	Cumulative yield (%)	Cumulative purification (fold)
Crude	100	1
DEAE	100	1
Heat	400	80
Mono Q	160	260
Affinity	60	ND ^a

^a ND, not done.

and *Xho*I sites of pBluescript-KS (Stratagene). Glyceraldehyde phosphate dehydrogenase (GAPDH) antisense RNA was produced from a construct containing the 1.3-kb *Pst*I fragment of the rat GAPDH cDNA in pBluescript (9). Antisense RNA for *c-myc* and GAPDH was generated by digestion of the plasmids with *Xba*I or *Mbo*II, respectively, and then by transcription in the presence of [³²P]UTP. RNA was harvested from transiently transfected P3X cells and DNase treated by the method of Sambrook et al. (32). Radiolabeled RNA probe was combined with 20 µg of sample RNA, heated to 85°C for 10 min, and hybridized overnight at 55°C. Samples were treated with RNase A and RNase T₁ at room temperature for 2 h; treatment was stopped by addition of SDS and proteinase K. Samples were phenol-chloroform extracted, precipitated, heated to 95°C for 3 min in sequencing dye, and separated by size on an 8% sequencing gel. Protected fragments were quantitated by use of a scanning beta counter.

RESULTS

Purified CF1 has an apparent molecular mass of 65 kDa. In order to determine the biochemical properties of CF1 and to assist in its identification, a purification protocol, using recognition of the *c-myc* -260 site in an EMSA to monitor CF1 protein, was developed. Proteins were extracted from rat or mouse liver nuclei by a modification of the protocol of Dignam et al. (5) and passed over a DEAE column to remove contaminating nucleic acids. Since CF1 binding activity proved to be heat stable, extracts were heated at 68°C to remove the bulk of contaminating proteins. CF1 was further purified by anion-exchange chromatography on an FPLC Mono Q column with linear salt gradient elution and by affinity chromatography on a CF1 oligonucleotide affinity column. The overall yield of CF1 activity was routinely in excess of 60% (Table 1).

Affinity-purified CF1 was separated by SDS-PAGE and stained with Coomassie blue to quantitate the proteins present in the preparation. A single 65-kDa protein could be detected by Coomassie blue staining, indicating this protein as the predominant protein in the preparation (data not shown). Gels were subsequently subjected to silver staining to detect proteins present in lower abundance. The 65-kDa protein was still a predominant protein, but additional proteins were also detected (Fig. 1A, lane 1). In order to determine which protein had CF1 binding activity, proteins from a preparative SDS-PAGE were eluted from gel slices and subsequently precipitated, denatured in guanidine, and renatured. When renatured proteins were analyzed by EMSA, only the gel segment containing the 65-kDa protein yielded CF1 binding activity (Fig. 1B, lane 3). The prevalence of the 65-kDa protein in the affinity-purified preparation and comigration of CF1 binding activity with the 65-kDa protein in SDS-PAGE suggest that the 65-kDa protein is CF1.

FIG. 1. Apparent molecular weight of CF1. Oligonucleotide affinity column-purified proteins were separated by SDS-PAGE in parallel lanes. One lane was stained to detect protein. The parallel lane was cut into slices, and the proteins were eluted, renatured, and subjected to EMSA analysis with a probe containing the -260 CF1 site. (A) The region comprised by each slice of the unstained gel is indicated to the left. Lane 1, proteins detected by silver staining; lane 2, protein standards of known molecular weight (MW) for comparison. (B) Results of EMSA analysis following protein renaturation. The slices numbered correspond to the slices indicated in panel A.

CF1 is the murine form of YY1. We had previously used cross-competition for binding and partial proteolysis to show that CF1 also bound to the µE1 site in the IgH enhancer (31). As we were completing the purification and identification of CF1, a protein, NF-E1, having a predicted molecular mass of approximately 40 kDa but which migrates in an SDS-polyacrylamide gel with an apparent molecular mass of 65 kDa, was cloned by Atchison and colleagues (1). This protein recognizes a sequence in the 3' enhancer of the human kappa light chain and also recognizes the µE1 binding site of the IgH enhancer (28). Sequence comparison had shown that NF-E1 was identical to another cloned protein, YY1, which recognizes two binding sites in the AAV P5 promoter (36). Comparison of the known CF1 binding sites with the binding sites recognized by YY1 (NF-E1) showed a strong degree of homology (Fig. 2A). YY1, like CF1, is ubiquitously expressed. In addition, YY1 is also stable at 68°C and its DNA-binding activity in an EMSA is strongly affected by NaCl concentration (21), as is that of CF1 (data not shown). The similarity in their mobilities on SDS-PAGE, the homology between their binding site consensus sequences, their ubiquitous distribution, and the similarities in their biochemical properties strongly suggested that CF1 might be the murine form of YY1.

Therefore, we utilized two approaches to test directly whether YY1 was identical to CF1. First, a monoclonal antibody against YY1 was tested for its ability to disrupt the complex formed by CF1 binding to the *c-myc* -260 site in an EMSA. This antibody is known to ablate the EMSA complex formed by YY1 on its recognition site (35a). As shown in Fig. 2B, increasing amounts of anti-YY1 or heterologous antibody were added to EMSA reaction mixtures containing purified CF1. Increasing amounts of the anti-YY1 antibodies were able to completely ablate the EMSA complex formed by CF1 (Fig. 2B, lanes 2 to 4). In contrast, heterologous monoclonal antibodies did not interfere with complex formation (Fig. 2B, lanes 6 to 8). This result indicates that YY1 and CF1 share a common antigenic epitope.

FIG. 2. CF1 and YY1 are highly similar. (A) Comparison between the -260 *c-myc* CF1 binding site and known YY1 recognition sequences. Letters in boldface indicate conserved nucleotides. The orientation of the sequence with respect to the start site of transcription is indicated (+, sense; -, antisense). (B) Recognition of CF1 by an anti-YY1 monoclonal antibody. Buffer alone (lanes 1 and 5) or increasing amounts (lanes 2 and 6, 0.2 μ l; lanes 3 and 7, 0.5 μ l; lanes 4 and 8, 1 μ l) of anti-YY1 (lanes 1 to 4) or heterologous (lanes 5 to 8) monoclonal antibody were added to binding reactions as indicated, allowed to bind, and subjected to EMSA analysis. (C) YY1 binding to the -260 *c-myc* CF1 recognition sequence. YY1 produced by in vitro translation was bound to probe containing the -260 *c-myc* CF1 binding site and subjected to EMSA (lane 1). Specific (μ E1) or heterologous (μ E3) oligonucleotide competitor (50 ng) was added to the binding reactions as indicated.

To further confirm the identity between CF1 and YY1, recombinant YY1 was produced by in vitro transcription and translation and its ability to recognize a *c-myc* CF1 binding site was examined. The in vitro-translated YY1 was used in an EMSA with a probe from the *c-myc* promoter containing both CF1 binding sites. YY1 bound to this probe, and the complex formed was specifically inhibited by an oligonucleotide corresponding to the IgH μ E1 site (Fig. 2C). Purified CF1 was also able to form a complex in an EMSA when an oligonucleotide comprising the P5+1 YY1 binding site from the AAV P5 promoter was used as probe (data not shown). Taken as a whole, the similarities in size and other physical properties between CF1 and YY1, their immunologic relatedness, and the ability of YY1 to recognize a CF1 binding site demonstrate that CF1 and YY1 are extremely similar proteins and support the view that CF1 is murine YY1. This view is also consistent with the widespread expression shown previously for CF1 (16) and YY1 (13). Accordingly, CF1 will henceforth be called mYY1 (murine YY1), and human YY1 will be referred to as hYY1. On the basis of these and previous (31) results, we conclude that YY1 also

FIG. 3. Cotransfection of YY1 and the *c-myc* promoter reporter construct. (A) Diagram of the YY1 coding region present in pCMV-hYY1. The box indicates the hYY1 cDNA sequence. The stippled region indicates the sequence encoding the zinc fingers, and the black box indicates the sequence deleted from pCMV-hYY1DZnF. (B) Diagram of the *c-myc* promoter region in pBBLuc. A 1.7-kb *Bgl*II-*Bgl*II fragment from the *c-myc* promoter controls expression of the luciferase reporter. The stippled region indicates the first exon of the *c-myc* gene, which is transcribed but not translated. The black box indicates the sequence deleted from pBBLucDN:X. The *Ava*I and *Hpa*II sites delineate the region used as a probe for EMSA analysis. (C) YY1 activates the *c-myc* reporter. A total of 10 μ g of pBBLuc and 10 μ g of pUC19 were cotransfected with 0.2 μ g of pCMV (lane 1), pCMV-hYY1 (lane 2), or pCMV-hYY1DZnF by electroporation. Cells were harvested after 12 h and assayed for luciferase activity by using a Berthold luminometer. Error bars indicate 1 standard deviation.

binds the CBAR site of the skeletal α -actin promoter. This has been demonstrated directly by Gualberto et al. and Lee et al. (10, 23).

hYY1 is a strong transactivator of the *c-myc* promoter. Since three binding sites for mYY1 had been identified in the *c-myc* promoter region (1, 12, 16, 31) and since a multimerized form of one of these sites had been shown previously to activate a heterologous promoter (31), we wished to test directly whether recombinant hYY1 was able to modulate transcription from the *c-myc* promoter. Accordingly, cotransfection experiments using a plasmid expressing hYY1 under control of the cytomegalovirus (CMV) promoter and enhancer (pCMV-hYY1 [Fig. 3A]) and a reporter containing the luciferase gene under the control of a fragment of the murine *c-myc* promoter extending from the *Bgl*II

site at -1150 nucleotides (from P1) to the *Bgl*II site at +565 nucleotides (pBBLuc [Fig. 3B]) were designed. The pCMV vector without insert was used as a control in parallel transfections. When pCMV-hYY1 was cotransfected with the pBBLuc reporter into plasmacytoma P3X cells by electroporation, expression of luciferase from the *c-myc* promoter was increased significantly (Fig. 3C, lanes 1 and 2). An expression vector containing a mutant form of hYY1 which lacks two-thirds of the zinc finger domain and cannot bind DNA (13), pCMV-hYY1DZnF, did not activate the *c-myc* promoter, indicating that the zinc finger region of hYY1 is necessary for the activation (Fig. 3C, lane 3). hYY1 activation of *c-myc* reporters is unusually sensitive to the growth state of the cells and is highest when the cells are growing in early log phase. Nonetheless, significant induction is always observed in P3X cells. Activation by hYY1 was also observed in M12 B-cell lymphomas and Swiss 3T3 fibroblasts (data not shown), demonstrating that activation was not unique to P3X cells.

Additional experiments were performed to characterize hYY1 activity on a variety of reporters in P3X cells. A CAT reporter construct, pBBCAT, containing the same *c-myc* promoter region as the luciferase reporter was also activated by cotransfection of hYY1 (data not shown). This demonstrates that the target of hYY1 activation is the *c-myc* promoter and also suggests that the effect is due to activation of transcription rather than stabilization of mRNA, since the bulk of the pBBLuc and pBBCAT mRNAs are different. Repression of a thymidine kinase promoter containing five GAL4 binding sites by a GAL4 1-147-YY1 fusion protein in P3X cells was similar to that previously observed in 3T3 and HeLa cells (36), demonstrating that YY1 can function in P3X cells as a repressor given the appropriate promoter context (Fig. 4A, compare GAL4-YY1 lanes 2 to 4 with control lanes 5 to 7 and 8 to 11). hYY1 does not affect transcription of a pSV2LUC reporter containing the simian virus 40 promoter and enhancer (Fig. 4B) or of a reporter dependent on the CMV promoter and enhancer (data not shown). Thus, YY1 does not have a general activating effect on transcription or activate via the luciferase coding sequence. Together, these results show that, in our experimental system, activation by hYY1 is specific for the *c-myc* promoter; other promoters tested were unaffected or repressed by hYY1.

A riboprobe assay was used to determine whether the hYY1-induced pBBLuc transcripts initiated at the normal *c-myc* transcript initiation sites. A *c-myc* reporter, pBBLucDN:X, containing a 181-bp deletion between the *Not*I and *Xho*I sites in exon 1, was used in these experiments; cotransfected YY1 activates this reporter as well as it activates pBBLuc (data not shown). A radiolabeled riboprobe corresponding to nucleotides -1150 to +516 of the *c-myc* promoter was hybridized with total RNA from P3X cells cotransfected with pBBLucDN:X and pCMV or pCMV-hYY1. The transcript produced by the transfected *c-myc* promoter differed from the endogenous transcript by the deletion of the 181 bp between the *Not*I and the *Xho*I sites; thus, transcripts originating from P2 of the transfected gene protect a fragment of 174 nucleotides, whereas transcripts from the endogenous gene protect a fragment of 356 nucleotides. As shown in Fig. 4C, lanes 2 and 3, hYY1 cotransfection resulted in increased levels of mRNA initiating at P2, the major promoter for most endogenous *c-myc* transcripts (20, 42). Quantitation of the protected *c-myc* fragments showed at least a 13-fold increase in the amount of RNA produced from the transfected *c-myc* promoter in the presence of hYY1. We were not able to determine whether

transcripts initiating at P1 were also increased, since the expected P1-protected fragment migrates in the same region as protected fragments from the endogenous *c-myc* transcripts. This analysis shows that cotransfected hYY1 causes an increase in transcripts initiated at the *c-myc* promoter P2.

We wished to determine whether YY1 had different effects on *c-myc* transcription, similar to the concentration-dependent effects of Kruppel, when present at low compared with high concentrations (33). A wide range of cotransfected hYY1—from 160 pg to 15 μ g—was tested for its ability to activate pBBLuc. As shown in Fig. 4D, activation by hYY1 showed a linear dose response between approximately 100 ng and 5 μ g of cotransfected hYY1 expression vector. Figure 4D shows that upon transfection of 4 ng or less of YY1 expression vector, no activation or repression was observed. pBBLuc has a high basal activity in the absence of cotransfected YY1; thus, repression, if it occurred, would have been easy to detect. We conclude that under our conditions, hYY1 is always an activator of the *c-myc* promoter.

Transactivation of the *c-myc* promoter by hYY1 is both binding site dependent and binding site independent. To determine whether cotransfected hYY1 activates *c-myc* promoter-based reporters by binding to known YY1 binding sites, several truncated and mutated *c-myc* reporters were tested by cotransfection with hYY1 (Fig. 5A). Low amounts of hYY1 expression vectors were used to ensure that all transfections were in the linear range, and multiple reporters were compared within a single experiment, enabling comparisons between different reporters. The pSNLuc reporter contains a 758-bp *Sma*I-*Not*I fragment of the murine *c-myc* gene which contains the -390 and -260 YY1 sites but lacks the +535 bp site (Fig. 5A). Cotransfected hYY1 activates this reporter well, and the amount of activation is not significantly different from that observed on the larger reporter pBBLuc under these transfection conditions (Fig. 5B). Therefore, hYY1 activation of the *c-myc* promoter does not depend on the +535 bp YY1 binding site or on any other *c-myc* gene sequences located between -1139 to -424 or +334 to +571 bp. Subsequently, site-directed mutations were made in the two 5' YY1 binding sites on the pSNLuc reporter at -390 and -260 bp to make the pmmSNLuc reporter (Fig. 5A). The activities of transfected pSNLuc and pmmSNLuc were not significantly different, confirming previous results (31) (data not shown). However, the ability of hYY1 to activate pmmSNLuc was decreased approximately 46% in cotransfections (Fig. 5B). Thus, we conclude that hYY1 transactivation of *c-myc* transcription is partially dependent on the YY1 binding sites at -390 and -260 bp. However, approximately half of the hYY1 transactivation appears to be independent of known YY1 binding sites. A smaller *c-myc* promoter construct, pANLuc, from an *Ava*I site to the *Not*I site (Fig. 5A), which contained only 473 bp of the *c-myc* regulatory region was also activated by hYY1 (Fig. 5B) to the same degree as pmmSNLuc, thus defining a minimal *c-myc* region responsive to YY1.

To determine whether this *Ava*I-*Not*I region might contain an additional YY1 binding site, overlapping restriction fragments covering the entire *Ava*I-*Not*I fragment were used as probes in EMSAs with recombinant and purified hYY1. No detectable YY1 binding was observed, even though strong binding was seen for a control probe containing the -260 YY1 site (data not shown). We estimate our assay would have detected binding which was 20 times lower than that seen for the -260 site. Thus, we conclude that cotransfected hYY1 can activate a *c-myc* promoter which lacks detectable YY1 binding sites.

Overexpressed hYY1 induces elevated *c-myc* transcripts from the endogenous gene. When reporters are introduced into cells by transient transfection, multiple copies present in each nucleus may titrate out endogenous transcription proteins. To avoid this problem, we determined whether overexpressed hYY1 was able to activate an endogenous *c-myc* gene, present in single copy per haploid genome. The CMV-hYY1 expression construct was transfected into several murine cell lines by using neomycin selection to obtain pools of stable transfectants. Some cell lines appeared to be killed by overexpression of hYY1, making it impossible to establish stable CMV-hYY1 lines. For instance, we were repeatedly unable to establish hYY1-expressing transfectants of human 293 cells which constitutively express ade-

FIG. 4. YY1 activates via the *c-myc* promoter. (A) GAL4-YY1 fusion protein represses a thymidine kinase promoter with upstream GAL4 binding sites. Cotransfections were performed by using CAT reporters dependent upon the thymidine kinase promoter (tk-CAT) or a thymidine kinase promoter with five GAL4 binding sites (G4-tkCAT) and CMV expression vectors for GAL4 1-147 (G4) or GAL4-1-147 fused with YY1 (G4-YY1). The amount (in micrograms) of DNA used for each cotransfection is shown. % conv., percent chloramphenicol acetylated. The experiment was carried out twice with similar results. (B) YY1 does not activate pSV2Luc. A total of 10 μ g of pSV2Luc and 10 μ g of pUC19 were cotransfected with 1 μ g of pCMV or 1 μ g of pCMV-hYY1. Error bars indicate 1 standard deviation. (C) Riboprobe analysis of *c-myc* reporter transcripts from transient cotransfections. Sample lanes represent RNA from P3X cells cotransfected with 10 μ g of pBBLucDN:X, 10 μ g of pUC19, and either pCMV (lane 1) or pCMV-hYY1 (lane 2). Size standards (lane 3) are radiolabeled fragments from *Hin*FI-digested pUC19. The bands representing probe protected by the transfected, P2-initiated RNA (arrow) and by GAPDH endogenous RNA (asterisks) are indicated. (D) Dose response of cotransfected YY1 expression vector for activating pBBLuc. Various amounts of pCMV-hYY1 (from 160 pg to 15 μ g) were cotransfected with pBBLuc into P3X cells; fold activation was determined by comparison to control cotransfections in which an identical amount of pCMV was used. Results represent three or more independent transfections, and error bars indicate ± 1 standard deviation.

novirus E1A and E1B (41a). However, with murine erythroleukemia cells, we were able to isolate pools of clones containing stably integrated CMV-hYY1. The reason for differences in the ability of cell lines to grow in the presence

FIG. 5. Activation of truncated and mutated *c-myc* promoters by YY1. (A) Diagram showing portions of the murine *c-myc* promoter which were present in the reporter constructs tested. Solid boxes indicate approximate location of known YY1 binding sites; numbers give position relative to P1. (B) Activation of *c-myc* promoters by cotransfected hYY1. P3X cells were cotransfected with 0.3 μ g of either pCMV (control) or pCMV-hYY1, 10 μ g of the indicated reporter, and 10 μ g of pUC19 DNA. Each value represents the average of at least six independent transfections; error bars, 1 standard deviation.

of overexpressed hYY1 is not understood, although it may depend upon differential expression of exogenous YY1 or different levels of endogenous proteins which respond to or interact with YY1.

Riboprobe analyses showed that hYY1 mRNA was present in the CMV-hYY1 pools but not in control CMV pools (Fig. 6). Riboprobe analyses were performed on control and CMV-hYY1 pools to compare the steady-state levels of *c-myc* mRNA transcribed from the endogenous *c-myc* gene. As shown in Fig. 6, *c-myc* mRNA levels in the pools which had elevated levels of exogenous hYY1 were higher than in control pools. Transcripts initiating at both P1 and P2 were elevated. When the autoradiograms were quantitated and normalized to the amount of control GAPDH mRNA present in each pool, the hYY1 pools were shown to have *c-myc* mRNA levels initiated from P2 4.5 ± 0.9 -fold higher than control pools. However, YY1 overexpression did not increase *c-myc* mRNA levels sufficiently to alter the ability of these cells to differentiate in response to dimethyl sulfoxide (35a). These results show that transcripts from the endogenous *c-myc* gene can be increased by elevated levels

FIG. 6. Overexpression of YY1 increases the amount of steady-state transcripts from the endogenous *c-myc* gene in MEL cells. Riboprobe analyses for steady-state mRNA levels expressed from the transfected hYY1 vector are shown in the top panel with a GAPDH control; the lower panels show endogenous *c-myc* mRNAs initiating from promoters P1 and P2, as indicated, with a GAPDH control. Lanes 1 and 2, control pools of pCMV stable transfectants; lanes 3 to 7, five pools of pCMV-hYY1 stable transfectants. Each pool represents 50 to 100 G418-resistant clones. Images shown of the P1-protected fragments were obtained by exposing the gel to film approximately three times longer than for the other protected bands.

of hYY1 and lend further support to the notion that YY1 is an activator of *c-myc* transcription.

DISCUSSION

We have demonstrated that the ubiquitous protein, common factor 1, appears to be identical to the zinc finger protein YY1 (NF-E1, delta, and UCRBP). This is consistent with previous reports showing that CF1 binds to the IgH μ E1 and skeletal α -actin downstream CBAR sites (31), which have both been shown (10, 23) to bind YY1. hYY1 is a strong transactivator of the *c-myc* promoter over a wide concentration range of cotransfected hYY1 expression vector concentrations. *c-myc* is the first promoter for which cotransfected hYY1 has been shown to be an activator, even though several YY1 binding sites appear to be activator sites. Curiously, approximately half of the response to YY1 is independent of detectable YY1 binding sites on the *c-myc* promoter. Using murine erythroleukemia cells stably transfected with a hYY1 expression vector, we have also shown that elevated levels of hYY1 cause increased levels of mRNA transcribed from the endogenous *c-myc* gene.

Mechanisms of hYY1 action on the *c-myc* promoter. The ability of hYY1 to activate transcription from *c-myc* promoter-based reporters and from an endogenous *c-myc* gene is intriguing because in GAL4 assays and in cotransfection assays using the AAV P5 promoter, hYY1 represses transcription (36). *c-myc* is the first promoter for which YY1 is clearly an activator, although YY1 binding sites in other elements, such as the IgH enhancer (24, 29, 39) and the ribosomal protein promoters (12, 13), have been shown by mutation to be activator sites. When cotransfection studies are carried out using these elements, YY1 may be found to activate them as well. Since hYY1-GAL4 fusion proteins have repressor activity in an assay in which binding is directed to GAL4 rather than YY1 binding sites (36), it

appears that in its simplest state, YY1 represses transcription. It is likely that other transcription factors which bind nearby sites or specific TATA-binding-protein-associating factors (6, 37) or other proteins (2, 19, 30) which participate in the formation of preinitiation complexes at the *c-myc* transcription initiation sites interact with YY1 in a way which alters its activity from a repressor to an activator.

It is intriguing that part of the hYY1 activation is independent of detectable binding sites within the *c-myc* promoter. It is formally possible that YY1 binds low-affinity sites within the minimal AN construct which were not detected by our binding assay. However, since strong binding was observed in EMSAs using a control probe with the -260 *c-myc* site, we estimate that the affinity of an undetected site would be at least 20-fold lower than that of the -260 site. Several alternative mechanisms could mediate binding site-independent activation by hYY1. Seto et al. (34) showed that hYY1 can act as a transcription initiator, and Shenk has recently found that hYY1 associates directly with TATA-binding protein (34a). Thus, by virtue of its association with TATA-binding protein, hYY1 could activate in a binding site-independent manner, although additional interactions would be necessary to render the effect promoter specific. Alternatively, hYY1 may act indirectly by binding to the regulatory element for a protein which in turn modulates *c-myc* transcription. Another intriguing possibility is that transfected YY1 interacts with an endogenous protein which regulates *c-myc* transcription. The binding site-independent activation of *c-myc* by YY1 may provide a key to better understanding of both *c-myc* regulation and YY1 function.

Adenovirus E1A has been found to reverse the ability of YY1 to repress other genes (36). E1A synergistically increases activation of *c-myc* by hYY1 (30a). Although the mechanism for this effect is not known, it suggests that E1A increases the activating ability of YY1 in promoters in which YY1 activates as well as in promoters in which YY1 represses. E1A interacts directly with YY1 (34a, 36a) and with TBP (22). It also activates *c-myc* by binding retinoblastoma protein and liberating the E2F activator (14, 25, 38). More work will be necessary to understand the mechanism(s) involved in synergistic activation of *c-myc* by YY1 and E1A.

Biological importance of YY1 activation of *c-myc*. The data presented here demonstrate that hYY1 can activate both exogenous and endogenous *c-myc* promoters when it is present at elevated levels. The dependence of part of the hYY1 activation upon the two 5' YY1 binding sites in the *c-myc* promoter is consistent with the general observation that sequence-specific DNA-binding proteins act by binding their target sequences. Although we previously reported that mutation of these sites did not decrease the activity of a CAT vector driven by a larger fragment of the *c-myc* promoter when assayed by transient transfection (31), the present data show that mutation of the -390 and -260 YY1 sites decreases the ability of cotransfected YY1 to activate a smaller *c-myc* reporter (Fig. 5). These data suggest, but do not prove, that YY1 normally activates *c-myc* transcription via the -390 and -260 binding sites. In the larger promoter construct, the effect of YY1 may have been small or redundant relative to those of other regulators. Similar apparent redundancy has been reported for the rpl30 YY1 site (12). Many aspects of *c-myc* transcriptional regulation have proved difficult to reproduce with transfected reporters (26), and it may be necessary to study *c-myc* transcription in mice in which the YY1 gene has been ablated to establish definitively a requirement for YY1 in *c-myc* transcription.

YY1 is expressed in many tissues in which it may provide

a constitutive rather than a regulated signal for *c-myc* activation. However, YY1 may be important for changing *c-myc* expression in some developmental situations such as myogenesis. *c-myc* levels fall when myoblasts differentiate into myotubes (7, 15). The importance of appropriate *c-myc* levels during this process is demonstrated by the fact that overexpression of *c-myc* prevents myogenesis (4, 7, 15, 27). Consistent with the observed decrease in *c-myc* expression, YY1 levels have been reported to decrease upon differentiation of primary myoblasts in culture and during muscle differentiation (21).

Adenovirus E1A protein has strong modulatory effects on many transcriptional regulatory elements (see reference 35 for a review), but its ability to amplify YY1's activation of *c-myc* may be an important aspect of E1A function. The natural role of E1A in infected cells is to stimulate exit from G₀ and to induce DNA replication. Activation of the *c-myc* gene appears to be important for reentry of cells into the cell cycle, and increasing the ability of YY1 to activate the *c-myc* promoter could be one of the ways in which E1A is able to force exit of infected cells from G₀. It may also be one reason why, when E1A is unable to promote virus replication in nonpermissive rodent cells, it can contribute to immortalization of these cells and cooperate in their oncogenic conversion in culture. Cellular homologs of E1A are likely to exist, and it will be interesting to determine whether these proteins also synergize with YY1 to activate *c-myc*.

ACKNOWLEDGMENTS

We thank Tom Shenk for the use of plasmid constructs, oligonucleotides, and antibodies produced in his laboratory. We thank H. Young and R. Dalla-Favera for critically reading the manuscript.

K.T.M. and K.-K.W. are supported by an MSTP training grant (GM 07367). This work was supported by USPHS grant CA 38571 to K.C.

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