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The Intracisternal A-Particle Upstream Element Interacts with Transcription Factor YY1 To Activate Transcription: Pleiotropic Effects of YY1 on Distinct DNA Promoter Elements

KAPAETTU SATYAMOORTHY,[1] KYOUNGSOOK PARK,[2] MICHAEL L. ATCHISON,[2] AND CHIN C. HOWE[1]*

The Wistar Institute of Anatomy and Biology, 3601 Spruce Street,[1] and Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania,[2] Philadelphia, Pennsylvania 19104

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Murine intracisternal A-particle long terminal repeats contain an intracisternal A-particle upstream enhancer (IUE) element that binds to a 65-kDa IUE binding protein (IUEB) present in both undifferentiated F9 embryonal carcinoma cells and differentiated parietal endoderm-like PYS-2 cells. This IUE element confers a CpG methylation-sensitive IUEB binding and enhancer activity. Using gel retardation, methylation interference, CpG methylation sensitivity binding, and cotransfection assays, we have now identified the 65-kDa IUEB as YY1 (also called NF-E1, δ, or UCRBP), a zinc finger protein related to the Krüppel family. YY1 binds to a number of similar but distinct DNA motifs, and cotransfection assays indicate that these motifs have different enhancer potentials in PYS-2 cells. The relative strengths of these elements are as follows: IUE > κE3′ from the human immunoglobulin kappa light-chain 3′ enhancer > upstream conserved region from the Moloney murine leukemia virus promoter. Results of DNA binding assays suggest that the differences in enhancer potentials are due to the different binding affinities of YY1 to the various motifs and the binding of two other transcription factors to the IUE sequence.

Murine intracisternal A particles (IAPs), which are defective retroviruses harbored in the cisternae of the endoplasmic reticulum and the Golgi complex of the host cell, are evolutionarily related to type B mouse mammary tumor virus, type D simian retroviruses, and type C avian sarcoma virus (for a review, see reference 19). Present in about 1,000 copies per haploid mouse genome, the full-length IAP type I genes are about 7 kb long, and the type II genes are about 4.8 kb long. IAP transcripts are prominently detected in germ cell oocytes, preimplantation embryos, and tumor cells such as plasmacytomas and neuroblastomas (18). Functionally, the IAP genes are known to transpose (15, 19) and affect expression of a number of cellular genes, including oncogenes, interleukins, and several growth factors (5, 19, 23).

We have used murine embryonal teratocarcinoma cells as a model system to analyze *cis* elements and *trans*-acting factors that regulate IAP gene expression. In the murine embryonal carcinoma cell line, F9, which resembles early embryonic cells (28, 36), IAP expression is restricted, whereas upon differentiation of these cells into parietal endoderm-like cells by treatment with retinoic acid and dibutyryl cyclic AMP, IAPs are highly expressed (16). This differential regulation of IAP gene expression is controlled primarily at the level of transcription (16). Previously we analyzed the IAP long terminal repeats that control transcription and identified a DNA element, located between nucleotides (nt) −53 and −47 within an IAP proximal enhancer. The element is active in differentiated parietal endoderm-like cells but inactive in undifferentiated F9 cells (20). We also found that an IAP upstream enhancer (IUE) element, located between nt −180 and −173, is active in both undifferentiated and differentiated cells (21). A 65-kDa IUE binding protein (IUEB) is present in both differentiated and undifferentiated cells. However, CpG methylation within the binding site inhibits both the IUEB binding and IUE transcriptional activities, suggesting that IAP expression may be regulated by binding site-specific methylation (21).

YY1 (35), also termed NF-E1, δ, or UCRBP (10, 13, 29), is a recently isolated transcription factor belonging to the Krüppel (33) superfamily of zinc finger proteins. YY1 was identified on the basis of its ability to bind to the adeno-associated virus P5 promoter P5-60 element (35). YY1 is encoded by the same gene as the NF-E1 protein, which binds to the immunoglobulin kappa light-chain 3′ enhancer (κE3′) element and the immunoglobulin heavy-chain enhancer μE1 site (29). YY1 corresponds to the upstream conserved region binding protein (UCRBP), which binds to the UCR element of the Moloney murine leukemia virus promoter (9, 10). YY1 is also the human homolog of the mouse δ protein, which binds to ribosomal protein gene promoters (13, 14). The YY1 binding sites within these elements resemble each other but are not identical. We found that the binding site for IUEB in the IAP gene promoter shares homology with the YY1 binding site within the κE3′ element and that IUEB and YY1 comigrate in a sodium dodecyl sulfate (SDS)-polyacrylamide gel as a 65-kDa protein, although YY1 has a predicted molecular size of 44 kDa as deduced from the amino acid sequence. In addition, although YY1 appears to function as a negative regulator of the κE3′, P5-60, and UCR elements, it acts as a positive regulator to the IUE site in a manner similar to the δ site in ribosomal protein promoters (10, 13, 21, 29, 35). In the present study, we showed that YY1 represents IUEB and acts to stimulate expression in murine PE-like PYS-2 cells. We found that the binding affinities of YY1 and the binding of additional factors to the various YY1 motifs appear to influence their levels of enhancer activities.

* Corresponding author.

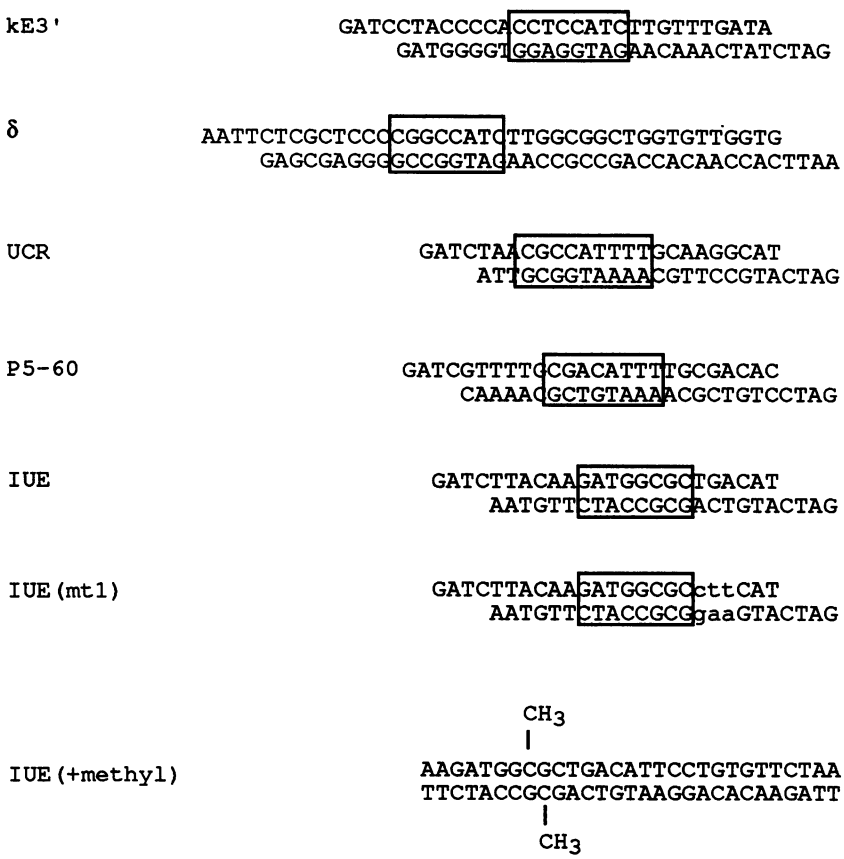

FIG. 1. Synthetic oligonucleotides containing various YY1 binding sites. The nucleotide sequences of the oligonucleotides and the YY1 binding sites (shaded areas) are from the following references: κE3', 29; δ, 13; UCR, 10; P5-60, 35; and IUE, 21.

MATERIALS AND METHODS

Plasmid constructs. Plasmids $(IUE)_4$TKCAT, $(\kappa E3')_4$TKCAT, and $(UCR)_5$TKCAT contain four copies of the IUE synthetic oligonucleotides, four copies of κE3', and five copies of UCR, respectively, inserted in front of the TK promoter in pBLCAT2 (24). Figure 1 lists the nucleotide sequences of each oligonucleotide. $(GAL4)_5$TKCAT (29) contains five copies of the GAL4 DNA-binding element upstream of the thymidine kinase (TK) promoter in pBLCAT2 and was a gift from Y. Shi (Harvard University). pSVGAL4 (1 to 147) is the previously described pSG424 (32), and pSVGAL4 (1 to 147)/YY1 is the previously described pSV-E1 (29), which consists of the YY1 cDNA inserted in frame at the *Eco*RI site of plasmid pSG424. pSV-YY1 (29) contains the YY1 cDNA cloned in the correct orientation in the pECE mammalian expression vector (6) under the control of the simian virus 40 promoter. CpG methylation of IUEB sites of the oligonucleotides or plasmids were performed using *Hha*I methylase (New England Biolabs), as described previously (21).

Preparation of nuclear extracts and YY1 synthesized in *Escherichia coli*. Nuclear extracts from PYS-2 cells were prepared in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.5 μg/ml), and pepstatin (0.7 μg/ml) as previously described (20, 21). YY1 was expressed in *E. coli* as a fusion protein containing six histidine residues by cloning the entire coding region of YY1 into the pET11d-6×His vector (kindly provided by T. Kadesch). The fusion protein was purified by nickel chelate affinity chromatography done according to the manufacturer's instructions (Qiagen). Bacterial (1-liter) cultures were grown to an optical density at 600 nm of 0.8 and then induced with isopropyl-β-D-thiogalactoside to a final concentration of 2 mM for 2 h. Cells were collected by centrifugation at 4,000 × *g* for 20 min and resuspended in 20 ml of 6 M guanidine hydrochloride, pH 8.0. After overnight incubation at 4°C, the supernatant was collected by centrifugation and processed by nickel chelate affinity chromatography, as described by the manufacturer. Protein was eluted at pH 4.5 and extensively dialyzed against successively decreasing amounts of guanidine hydrochloride in phosphate-buffered saline.

Gel retardation assays. The assays were performed as previously described (20, 21), with modifications in the amount of extract proteins to enhance binding of minor proteins. Briefly, a 10-μl reaction mixture containing 14 μg of nuclear extract protein and 1 μg of double-stranded poly(dI-dC) was incubated for 15 min at 30°C in binding buffer consisting of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9), 60 mM KCl, and 5% glycerol. After the addition of 50,000 cpm (0.15 to 0.2 ng) of the probe, reaction mixtures were incubated for another 15 min. DNA-protein complexes were resolved on 5% polyacrylamide gels. Probes were prepared by 5' or 3' end labeling of annealed double-stranded oligonucleotides with T4 polynucleotide kinase and [γ-^{32}P]ATP or the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP, respectively. Gel retardation assays with purified YY1 were per-

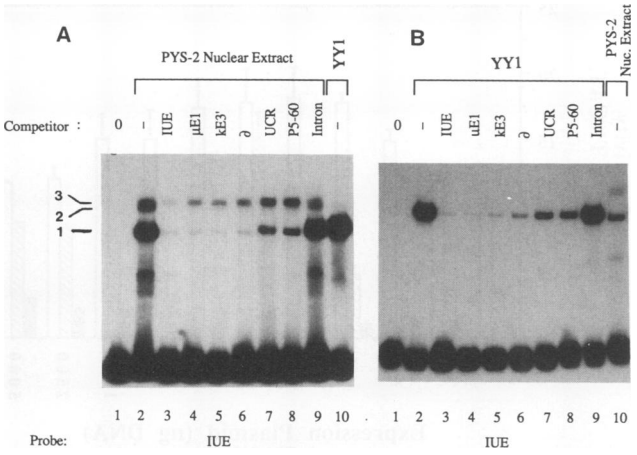

FIG. 2. DNA binding of PYS-2 nuclear proteins (A) and cloned YY1 synthesized in *E. coli* (B) to IUE. The source of each protein (PYS-2 nuclear extract or cloned YY1) and the oligonucleotide competitors (15 ng) are indicated above each lane (see Fig. 1 for nucleotide sequences of the competitors). The positions of the major DNA-protein complex 1 and two minor complexes, 2 and 3, which are evident in lane 10 of panel B, are also indicated.

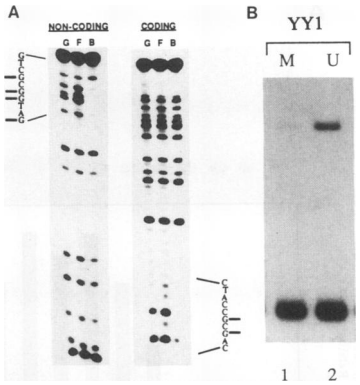

FIG. 3. YY1 binding site sequence within IUE and CpG methylation-sensitive binding characteristics. (A) Methylation interference analysis of noncoding and coding strands. The piperidine cleavage patterns for the probe not incubated with extract (G, lanes 1 and 4) and for the free (F, lanes 2 and 5) and bound (B, lanes 5 and 6) probes are shown. The methylated G residues from nt −180 to −173 that interfered with the DNA-protein interaction are indicated. (B) Effects of CpG methylation on YY1 binding. The CpG methylation of IUE with *Hha*I methylase and testing of completeness of methylation (not shown) were carried out as previously described (12). Lanes 1 and 2, binding of YY1 to methylated (M) and unmethylated (U) IUE probes, respectively.

formed under the same conditions as described for nuclear extracts, except that approximately 5 ng of YY1 and 0.25 μg of poly(dI-dC) were used in reaction mixtures, and incubations were performed at 25°C. For competition experiments, excess unlabeled competitor DNA in various amounts was preincubated with nuclear extracts or purified YY1 for 15 min prior to probe addition. To study the requirement of Zn^{2+} for YY1 binding, 1,10-phenanthroline (0.5 mM) (Sigma) was preincubated with nuclear proteins 15 min prior to probe addition.

Methylation interference and transfection assays. These assays were performed as previously described (20, 21). Typically, 4 μg of reporter plasmid and 4 μg of TK-human growth hormone (as an internal control for transfection efficiency) were used in transient transfection assays with calcium phosphate precipitates. Amounts of expression constructs used in cotransfection analyses are indicated in the figure legends. The total amount of transfected DNA was adjusted to 14 μg with pUC18.

UV cross-linking analysis. 5′-Bromo-2′-deoxyuridine (Sigma)-substituted duplex oligonucleotide probes of IUE and IUE (mt1) were prepared as described previously (21). The binding reaction mixtures were scaled up 30- to 40-fold under the conditions mentioned above for nuclear proteins and subjected to UV cross-linking analysis essentially as previously described (21). The cross-linked protein-DNA complexes were separated on SDS–7 or 10% polyacrylamide gels and dried prior to autoradiography.

RESULTS

Identification of YY1 as the protein that binds to IUE. To determine whether YY1 corresponds to the IUEB protein, we examined and compared the binding specificities of these proteins. Binding of recombinant YY1, purified from a bacterial extract, to the IUE probe resulted in a DNA-protein complex band with electrophoretic mobility identical to that of the major complex produced from the binding of IUE to the 65-kDa endogenous IUEB in PYS-2 nuclear extracts (Fig. 2, complex 1, lanes 2 and 10). The significance of the other two complexes (complexes 2 and 3) observed with the PYS-2 nuclear extracts will be discussed below. The IUE-YY1 complex and the major IUE-nuclear protein complex showed identical competition patterns with unlabeled oligonucleotides containing various YY1 binding sites (Fig. 1); the IUE, μE1, κE3′, and δ elements were efficient competitors, whereas the UCR and P5-60 elements were relatively inefficient. An unrelated sequence (intron) (20) did not compete for the binding. Thus, YY1 binds the IUE element with the same specificity as that of the PYS-2 nuclear IUEB factor.

Methylation interference assays to analyze the sequences within IUE recognized by YY1 showed that methylation of guanine (G) residues at nt −174, −176, −177, and −180 on the noncoding strand and nt −175 and −173 on the coding strand interfered with the DNA-protein interaction (Fig. 3A). These are the same protein contact sites identified in methylation interference assays with endogenous IUEB (21). Therefore, YY1 binding to the sequence GATGGCGC, located between nt −173 and −180, is identical to the binding site sequence for the IUEB protein present in PYS-2 cells.

CpG methylation of the *Hha*I site within the IUEB binding site inhibits binding of the IUEB (21). Likewise, CpG methylation of the same site specifically reduced the binding of YY1 (Fig. 3B). Thus, on the basis of binding specificity, binding site sequence, and methylation-sensitive binding characteristics, the factor responsible for the formation of the IUE-protein complex appears to be YY1.

Transcription activation by YY1. We previously showed that IUE and its binding factor(s) are involved in positive regulation of a reporter gene (21). To further confirm the identity of YY1 as the IUEB, we analyzed the ability of YY1 to activate transcription of a target gene. To overcome the effect of the endogenous protein, YY1 was expressed as a fusion protein with the GAL4 DNA-binding domain [GAL4 (1 to 147)] under the control of the simian virus 40 promoter from the plasmid pSVGAL4/YY1. This plasmid was cotrans-

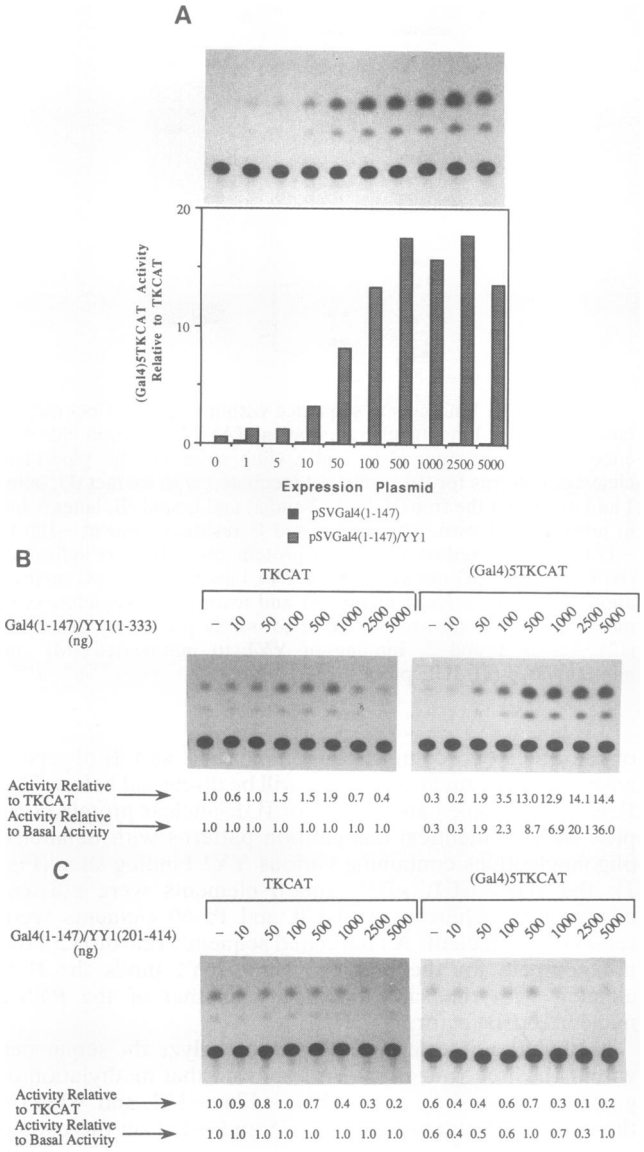

FIG. 4. YY1-activated transcription of a reporter TKCAT gene containing GAL4 binding sites. (A) PYS-2 cells were cotransfected with 4 μg of the reporter $(GAL4)_5$TKCAT and the indicated amounts of expression plasmids. The upper panel shows the autoradiograms of reaction products from CAT assays. The lower panel illustrates the CAT activity relative to TKCAT activity set at 1. ■, pSVGAL4 (1 to 147); ▨, pSVGAL4 (1 to 147)/YY1. Results are the averages of three independent transfections, and standard deviations did not exceed 16%. (B and C) PYS-2 cells were transiently transfected with $(GAL4)_5$TKCAT and the indicated amounts of deletion mutants of GAL4 (1 to 147)/YY1. The reporter plasmid, TKCAT, cotransfected with various concentrations of GAL4 (1 to 147)/YY1 deletion mutants are also shown. Levels of CAT activity expressed as percentages of acetylated [^{14}C]chloramphenicol and relative to TKCAT are indicated and are the averages of two independent experiments.

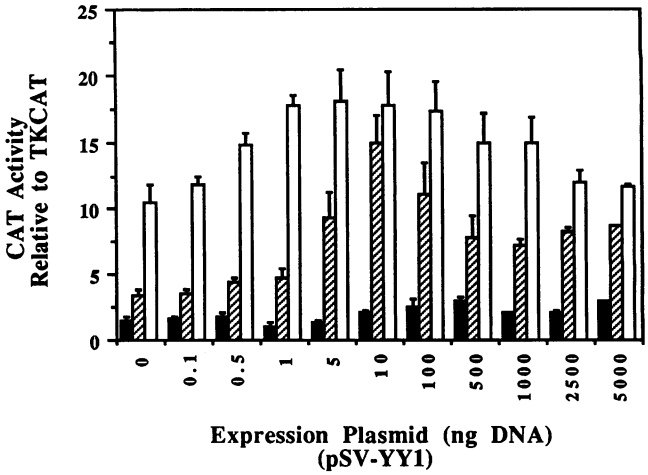

FIG. 5. Transcriptional effect of YY1 on various YY1 binding sites. PYS-2 cells were cotransfected with 4 μg of reporter plasmids $(IUE)_4$TKCAT (□), $(\kappa E3')_4$TKCAT (▨), and $(UCR)_5$TKCAT (■) and increasing amounts of the pSV-YY1 expression plasmid. Cells were harvested 48 h posttransfection to determine CAT activities. Results are averages of at least three independent transfections, and standard deviations are shown. The activities were determined by thin-layer chromatography.

fected into PYS-2 cells with a TKCAT plasmid containing GAL4 DNA-binding sites. As shown in Fig. 4A, increasing the amount of the GAL4/YY1 fusion protein markedly increased chloramphenicol acetyltransferase (CAT) activity from the $(GAL4)_5$TKCAT reporter plasmid; the level of CAT activity increased 18-fold with the addition of 500 ng of the expression plasmid. Control experiments showed that this YY1 activity was not due to the effect of the GAL4 DNA-binding domain, since pSVGAL4 (1 to 147) had no effect on expression of the reporter gene. We examined the N (amino acids 1 to 333)- and C (amino acids 201 to 414)-terminal regions of YY1 for influences on gene expression. The N-terminal region (amino acids 1 to 333) fused to the GAL4 DNA-binding domain [GAL4 (1 to 147)/YY1 (1 to 333)] activated transcription from $(GAL4)_5$TKCAT (Fig. 4B). However, GAL4 (1 to 147)/YY1 (201 to 414) did not activate or repress transcription from the GAL4 binding site in $(GAL4)_5$TKCAT compared with the level of TKCAT expression (Fig. 4C). These results indicate that YY1 acts as a transcription activator in PYS-2 cells and the activation domain lies within the N-terminal 333 amino acids. The slight decrease in CAT activity at high levels of GAL4/YY1 (201 to 414) fusion protein was not due to repressor function of YY1, because the same level of reduced expression was observed in the absence of GAL4 binding sites (Fig. 4C). In fact, control experiments indicated that the decrease is, in part, due to titrating out of factors by the simian virus 40 promoter in the expression vector. Thus, YY1 acts as a positive factor in PYS-2 cells, in agreement with the previous functional analysis of the IUEB protein (21).

We also analyzed the ability of YY1 to stimulate transcription from the intact IUE sequence. We constructed a plasmid, pSV-YY1, to express the native YY1 cDNA from the simian virus 40 promoter, without the GAL4 DNA-binding domain. This expression plasmid was cotransfected into PYS-2 cells with a TKCAT reporter plasmid containing the IUE segment, which contains the binding site for the IUEB protein (Fig. 5). As in our previous results, in the absence of pSV-YY1, the endogenous IUEB itself leads to a significant increase in CAT activity (21). Addition of pSV-YY1 resulted in a further increase in the levels of CAT activity. The extent of enhancement in CAT activity by pSV-YY1 is not great, being only 1.5-fold at best, which was achieved at 5 ng of the

TABLE 1. Methylation-dependent transcriptional activity mediated by YY1 in vivo[a]

Reporter plasmid	CpG methylation	YY1	Relative CAT activity ± SD
TKCAT	−	−	1.0
TKCAT	−	+	0.8 ± 0.01
PYF101TKCAT	−	−	16.4 ± 3.3
PYF101TKCAT	−	+	16.0 ± 2.1
$(IUE)_4$TKCAT	−	−	13.6 ± 1.3
$(IUE)_4$TKCAT	−	+	18.4 ± 3.1
TKCAT	+	−	1.2 ± 0.06
TKCAT	+	+	0.9 ± 0.1
PYF101TKCAT	+	−	17.6 ± 3.0
PYF101TKCAT	+	+	17.4 ± 3.8
$(IUE)_4$TKCAT	+	−	2.4 ± 0.08
$(IUE)_4$TKCAT	+	+	2.4 ± 0.2

[a] Transient transfections were performed with PYS-2 cells as mentioned in Materials and Methods by using the indicated CpG-methylated (with *Hha*I-methylase) or unmethylated reporter plasmids in the presence or absence of 5 ng of pSV-YY1 expression plasmid. Results are the mean of two independent experiments.

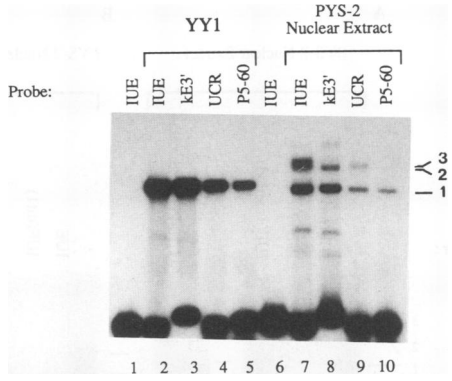

FIG. 6. Gel retardation assays performed with YY1 or PYS-2 nuclear extract and various ^{32}P-labeled DNA probes as indicated above the lanes. The positions of complexes 1, 2, and 3 are indicated on the right. The amount of factor bound to the DNA probes was quantified by an Ambis radioimaging system from the dried gels.

expression plasmid. The 5 ng of pSV-YY1, compared with the 500 ng of pSVGAL4 (1 to 147)/YY1 required to achieve the highest enhancement in CAT activity, also indicates the abundance of the endogenous IUEB in PYS-2 cells.

Since YY1 binds to several motifs that are similar to but distinct from the IUE sequence, we analyzed the ability of YY1 to stimulate transcription from other YY1 binding elements (Fig. 5). pSV-YY1 stimulated CAT activity more efficiently from the κE3′ enhancer sequence (about 4-fold) than from the IUE motif (1.5-fold), although the κE3′ sequence is not as potent an enhancer as the IUE element in the absence of pSV-YY1. However, as in the case of IUE, use of higher concentrations of pSV-YY1 resulted in decreased CAT activity from the κE3′ sequence. Neither pSV-YY1 nor the endogenous factor significantly stimulated or repressed CAT activity from the UCR motif at the concentrations of effector plasmid used. Thus, it appears that various YY1 motifs not only possess different degrees of enhancer potential by binding to the endogenous protein(s) in PYS-2 cells but are also activated to different extents by the additional YY1 supplied by the expression plasmid.

Methylation of IUE abolishes transcription in vivo. As shown in Fig. 3B, the unmethylated IUE element binds to YY1, while methylation of the CpG dinucleotide in the YY1 binding site greatly reduces the binding. We examined in vivo whether CpG methylation inhibits the YY1 activity (Table 1). As shown in Fig. 5, transfection assays with PYS-2 cells showed that CAT activity of the unmethylated reporter plasmid $(IUE)_4$TKCAT was activated by the endogenous factor and further activated by the 5 ng of the cotransfected plasmid expressing YY1 (Table 1). Methylation of the YY1 binding site in the reporter plasmid not only abolished CAT activity by the endogenous YY1, but also that by the exogenously added YY1 (Table 1). In a control experiment, *Hha*I methylation of a plasmid with a mutant polyoma-virus enhancer, PYF101 (20), driving the TK promoter had little effect on transcriptional activity (Table 1, rows 3, 4, 9, and 10), indicating that the transcriptional repression by methylation is specific for the YY1 binding site.

Characterization of protein binding potentials of IUE, κE3′, and UCR DNA sequences. Although the IUE, κE3′, and UCR elements all bind to YY1, our results above indicate that they show considerable differences in their abilities to drive CAT expression in PYS-2 cells. These differences might be due to the presence of cellular proteins other than YY1, which bind independently to nucleotide sequences outside the YY1 binding site. Examination of nuclear factors binding to IUE revealed two minor DNA-protein complexes (Fig. 2A and 6, bands 2 and 3) in addition to the major YY1 (or IUEB) band. Factor 2 in IUE-protein complex 2 also binds to the κE3′ and UCR sequences, but less efficiently (Fig. 6, lanes 7 to 9). Complex formation was inhibited by the unlabeled respective oligonucleotides (results not shown), indicating that the binding was specific. Experiments in which formation of IUE-protein complex 2 was abolished by competition with various YY1 binding oligonucleotides (Fig. 2A) confirmed the binding of factor 2 to the respective oligonucleotides. In contrast, the factor in IUE-protein complex 3 appears to bind only to the IUE sequence, as indicated by binding assays (Fig. 6, lanes 7 to 10) and competition analyses in which formation of complex 3 was abolished only by the IUE sequence (Fig. 2A). Together, these results suggest that high enhancer potential of the IUE sequence compared with that of other sequences could be due to the binding of factors 2 and 3 in addition to YY1, whereas only factor 2 together with YY1 interacts with the κE3′ and UCR oligonucleotides.

To determine whether factors 2 and 3 contact sequences flanking the YY1 binding site, we synthesized mutant IUE oligonucleotides containing methyl groups at the *Hha*I site [IUE(+methyl) (Fig. 1)] or mutations [IUE(mt1) (Fig. 1)] within the TGAC sequence which flanks the YY1 binding site and corresponds to the half-site of the consensus cyclic AMP response element (CRE). In competition experiments (Fig. 7A), bindings of YY1 and factor 2 but not factor 3 to the IUE oligonucleotide were efficiently inhibited by the IUE(mt1) oligonucleotide, while bindings of factors 2 and 3 but not YY1 to the IUE probe were abolished by the IUE(+methyl) oligonucleotide. These results suggest that factor 2 and 3 binding sites do not share the specific nucleotides needed for YY1 binding and that factor 3 requires the TGA sequence for binding. Addition of the zinc chelator 1,10-phenanthroline inhibited the binding of the YY1 zinc finger protein to the IUE sequence but not the bindings of factors 2 and 3, suggesting that factors 2 and 3 do not require zinc for their binding and thus are probably not

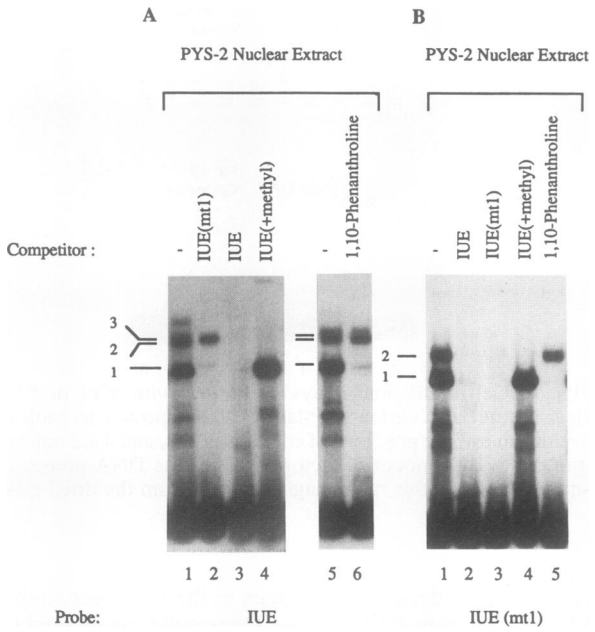

FIG. 7. Characterization of proteins that bind to IUE. PYS-2 nuclear proteins were incubated with a 200-fold excess of indicated competitors and subsequently allowed to bind to ^{32}P-labeled IUE (A) or IUE(mt1) (B) DNA oligonucleotides. 1,10-Phenanthroline was used at a final concentration of 0.5 mM. The positions of YY1 complex (complex 1) and complexes 2 and 3 are indicated to the left of the panels.

zinc finger proteins. These results also indicate that factors 2 and 3 can bind to DNA independently of the binding of YY1.

Observations that factor 2 and 3 binding sites differ from the YY1 binding site are confirmed by binding of IUE(mt1) oligonucleotide to PYS-2 nuclear extract (Fig. 7B). The binding yielded YY1 complex (complex 1) and complex 2 but failed to form complex 3. Competition with oligonucleotides methylated at the *Hha*I site alleviated complex 2 formation, but not YY1 complex formation. The zinc-chelating agent

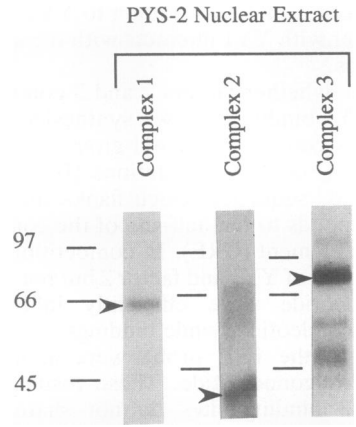

FIG. 8. UV cross-linking analysis of protein complexes bound to IUE. Proteins bound to bromodeoxyuridine-substituted ^{32}P-labeled IUE DNA probe were isolated and separated on SDS-polyacrylamide gels as described in Materials and Methods. The molecular mass (in kilodaltons) corresponding to each protein complex is indicated.

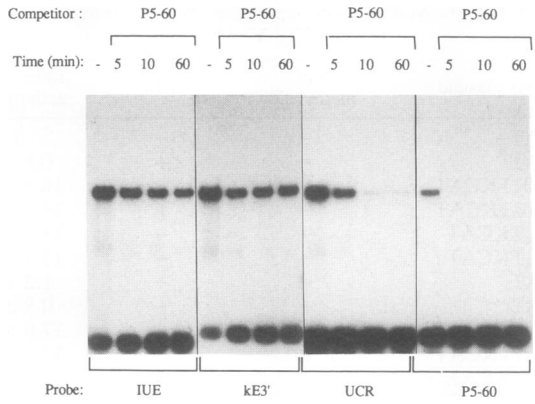

FIG. 9. Stability of YY1 binding to ^{32}P-labeled DNA elements. DNA probes were incubated with YY1 for 40 min to allow formation of DNA-protein complexes. At various times before samples were applied to the gel, a 200-fold excess of unlabeled competitor oligonucleotide P5-60 was added and incubation continued. Samples not inhibited by P5-60 (−) were included as controls. Bound YY1 was quantified with an Ambis radioimaging system.

again abolished binding of YY1 but not binding of factor 2. That factors 2 and 3 differ from each other and from YY1 is also evident from UV cross-linking analyses (Fig. 8). Factor 2 has a molecular mass of 40 kDa, while the major factor 3 is an 80-kDa protein, compared with YY1, which is of 65 kDa.

Another mechanism that might influence the enhancer potential of different YY1 binding motifs involves their binding affinities. As shown in Fig. 2, both UCR and P5-60 weakly competed for binding of endogenous or purified YY1 to the IUE sequence, indicating that the binding affinity of YY1 to the respective motifs differs. Analysis of the binding of YY1 or PYS-2 nuclear extract proteins to IUE, κE3′, UCR, and P5-60 sequences (Fig. 6) revealed the following order of steady-state binding capacities of these probes: IUE = κE3′ > UCR > P5-60. Quantitative analyses of the DNA-protein complexes indicated that the binding affinities of UCR and P5-60 were about 2.0- and 3.5-fold-less strong, respectively, than to the IUE or κE3′ sequences when either purified or endogenous YY1 was used (data not shown). Thus, UCR and P5-60 have lower binding affinities to YY1 than do IUE and κE3′.

The stability of the complexes involving YY1 binding to IUE, κE3′, UCR, and P5-60 sequences was examined to confirm their binding characteristics. Labeled IUE, κE3′, UCR, and P5-60 were allowed to bind to YY1 and were subsequently inhibited with an excess of unlabeled P5-60 DNA for various times (Fig. 9). The IUE and κE3′ elements formed a stable complex with YY1, with a dissociation rate (expressed as half-life) of nearly 55 min, compared with about 10 and 2 min for UCR and P5-60, respectively, suggesting relatively unstable complexes.

DISCUSSION

We demonstrated previously that activation of IAP expression during differentiation of F9 cells correlates with the binding of a 65-kDa IUEB to the IUE sequence and that CpG methylation within the IUEB binding site inhibits both IUEB binding and IUE transcriptional activity (21). We now show that on the basis of its binding site sequence, electrophoretic mobility in band shift assays, CpG methylation-sensitive binding characteristics, and functional role, the 65-kDa

protein is very likely the transcription factor YY1 (NF-E1, δ, or UCRBP) (10, 13, 29, 35). We have analyzed the binding properties of YY1 to the IUE motif, as well as to other YY1 binding sequences that share similar, but not identical, nucleotide sequences. These binding studies indicate that the IUE and κE3′ motifs bind to YY1 with the highest affinity to form the most stable complex followed by the UCR and P5-60 sequences. A structural study of the mouse immediate-early protein, Zif268, shows that each of the three zinc fingers makes direct contact with two G residues in the major groove of the DNA (17, 30), indicating that the more G residues in the binding site, the more stable the resulting complex. Concurrently, the IUE and κE3′ sequences, which show a high affinity to YY1, have more G residues than the UCR and P5-60 sequences, which have a low affinity to YY1. In addition, both the UCR and P5-60 motifs contain short dT stretches known to induce DNA bending (4, 25, 37), which may lead to imperfect DNA-protein contacts and thus low binding affinity. This hypothesis will be tested by a structural study of the complexes.

We also compared the enhancer activity of oligonucleotides containing various YY1 binding motifs from different promoters. Our transfection assays indicate that these (various YY1 motif-containing) oligonucleotides have different enhancer potentials in PYS-2 cells. These differences in enhancer potential appear to result from the combined effects of (i) differential binding affinities and stabilities of YY1 to the motifs and (ii) additional factors that bind outside of the motifs. For instance, the IUE oligonucleotide showed the highest enhancer activity, followed by the κE3′ sequence, while the UCR sequence, which showed the weakest binding to YY1, also showed the lowest enhancer activity in PYS-2 cells. However, while the YY1 binding characteristics of the IUE and κE3′ sequences are quite similar, the IUE sequence showed the greatest functional activity. This difference in enhancer activity between the IUE and κE3′ sequences may be explained by the ability of the IUE sequence to bind to additional nuclear factors. In addition to YY1, two PYS-2 nuclear factors (factors 2 and 3) bind independently to their own recognition sequence within the IUE sequence to yield minor complexes 2 and 3. On the other hand, the κE3′ and UCR sequences bind to YY1 and factor 2, but not to factor 3. Therefore, the elevated enhancer activity of the IUE sequence could be due to factor 3 binding to its contact site and activating transcription either directly or by modulating YY1 function. Factor 3, which is 80 kDa in size, is neither CREB nor Jun. IUE binding to two cellular proteins in addition to YY1 could also explain the relatively low enhancement of CAT activity by pSV-YY1 in cotransfection assays. That is, addition of pSV-YY1 increased the amount of YY1 in transfected cells but not the amount of the other cellular proteins that also bind to IUE to transactivate the reporter gene. Thus, the amount of available factors could limit the extent of CAT activity enhancement in cotransfection assays.

Functions of sequences containing various YY1 binding motifs vary not only from promoter to promoter within a cell type, but also from cell type to cell type. In the present study with GAL4-YY1 fusion proteins on a GAL4-driven promoter, YY1 acts to increase expression in PYS-2 cells. This is in discord with the previously reported repressor activity of YY1 in HeLa and 3T3 cells with the very same GAL4-containing effector and reporter plasmids (29, 35). In fact, we did not observe enhancer activity with the same effector and reporter plasmids in HeLa cells (data not shown). The reason for these diverse effects by YY1 in different cell types remains to be determined. However, in view of the fact that no transcription factor binding site other than the GAL4 binding site is available upstream of the TK promoter in the reporter plasmid, one reasonable speculation is that YY1 interacts directly with a PYS-2 cellular factor which differs from HeLa and 3T3 cellular factors. This speculation will also accomodate cell-type-specific differences in domain functions as revealed by GAL4 fusion proteins in transfection assays. For example, in PYS-2 cells, we found that the amino-terminal portion of YY1 contains the transactivation function, while in HeLa cells (35) the carboxy-terminal portion is necessary for the repressor function and the amino-terminal portion has neither activation nor repressor activity.

In addition to the possible existence of cell-type-specific YY1-associated factors, it is also possible that additional cell-type-specific factors bind to sequences flanking YY1 motifs. The binding of these additional factors may provide a mechanism for an ever-growing number of promoters, containing various YY1 binding motifs and flanking sequences, to display different functions. These include the YY1 binding sequences in the adeno-associated virus P5 promoter, the BZLF1 promoter of Epstein-Barr virus, the murine leukemia virus long terminal repeat, the c-*fos* serum response element, the α-actin muscle response element, or the human γ- and ε-globin genes, which appear to repress transcription (10–12, 22, 26, 29, 35). On the contrary, the YY1 binding region in the ribosomal protein L30 gene, the insulin promoter, the c-*myc* promoter, and the immunoglobulin heavy-chain enhancer element μE1 appear to function in a positive sense to activate transcription (13, 14, 27, 31). In addition, the YY1 binding site in the adeno-associated virus P5 promoter mediates adenovirus E1A protein inducibility of transcription (35). Moreover, YY1 can function as an initiator sequence-binding protein that directs transcriptional activation (2, 34).

Specific methylation of cytosine residues at CpG sites in mammalian DNA is thought to play a role in tissue- and development-specific gene expression (reviewed in references 1 and 3). CpG methylation, which regulates IAP gene expression, has been elucidated in several studies (7, 8, 21). We have now identified YY1 as a transcription factor that confers methylation-sensitive, transcriptional activity. Although YY1 is present in both differentiated and undifferentiated cells, we demonstrated that it binds very poorly to DNA methylated at CpG sites. Thus, activation of IAP expression during F9 cell differentiation may well be due to changes in the methylation state, from hypermethylated in undifferentiated F9 cells to hypomethylated in differentiated cells. Similarly, although YY1 is present at all stages of development, methylation of the YY1 DNA-binding site could provide a mechanism for affecting accessibility of the factor to DNA. Interestingly, four of the five YY1 binding sites listed in Fig. 1 contain CpG dinucleotides. It will be interesting to determine whether methylation of these binding sites also influences the ability of YY1 to bind to DNA.

In conclusion, diverse mechanisms, including CpG methylation of the YY1 binding site, differences in binding affinities of YY1 to DNA motifs, and additional factors binding to the DNA motifs, appear to mediate the functional versatility of YY1 in the regulation of a number of genes and perhaps differentiation and development.

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