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Functional Domains of the Transcription Factor USF2: Atypical Nuclear Localization Signals and Context-Dependent Transcriptional Activation Domains

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USF is a family of basic helix-loop-helix transcription factors that recognizes DNA-binding sites similar to those of the Myc oncoproteins. Here, various functional domains in the mouse USF2 protein were identified and characterized. Indirect immunofluorescence studies with transiently transfected cells revealed that both the basic region and the highly conserved USF-specific region (USR) are involved in the nuclear localization of USF2. Cotransfection assays with deletion mutants containing the DNA-binding domain of either USF2 or GAL4 identified two distinct transcriptional activation domains in USF2, the USR and the exon 5-encoded region. Activity of the exon 5 activation domain was detectable in both assay systems. Within USF2, however, its potency varied with the conformation induced by the surrounding regions, especially that encoded by alternatively spliced exon 4. In contrast, the USR activated transcription only in its natural context upstream of the USF2 basic region and only with reporter constructs containing the adenovirus major late minimal promoter but not the E1b minimal promoter. However, insertion of an initiator element downstream of the TATA box rescued the activity of the USR on E1b-driven reporters. The USR therefore represents a new type of activation domain whose function depends very strongly on the core promoter context.

Transcription factor USF was originally identified by its ability to bind to the adenovirus major late (ML) promoter and stimulate transcription in vitro (3, 31, 42). In HeLa cells, USF was shown to consist of two polypeptides with apparent molecular masses of 43 and 44 kDa (41, 43). cDNA clones encoding these two proteins, termed, respectively, USF1 and USF2, were isolated from both humans and mice (14, 45, 46), and other family members were subsequently cloned from sea urchins and *Xenopus laevis* (18, 22). Analysis of these clones demonstrated that USF belongs to the Myc family of regulatory proteins characterized by a C-terminal basic-region (BR)–helix-loop-helix (HLH)–leucine zipper (zip) structure responsible for dimerization and DNA binding (16, 32). The different USF family members are all extremely similar in the BR-HLH-zip domain, while the N-terminal regions, which are possibly involved in transcriptional activation, are highly divergent (45, 46).

USF recognizes sites on the DNA that contain a CACGTG core sequence. Cocrystallization of the C-terminal DNA-binding domain of human USF1 with its specific DNA-binding site revealed that USF dimers bind DNA as a four-helix bundle, with the basic domain from each monomer contacting half of the DNA-binding site (10). Transcriptional activation by USF can be demonstrated both in vitro and in vivo (9, 21, 25, 35, 38, 42), and the possible involvement of USF in the transcriptional regulation of many different genes has been suggested. Unfortunately, since the putative USF target sequences may also be recognized by several other BR-HLH-zip proteins in vivo, it has been difficult to assess the direct involvement of USF in the regulation of these genes. Recently, the development of dominant-negative mutants of USF has provided a means to address this question. USF mutants that are capable of dimerization but fail to bind DNA were introduced into cells to test the role of endogenous USF in the regulation of gene expression. In two cases, transcriptional activation by varicella-zoster virus immediate-early protein IE62 (29) and regulation of the glucose response in hepatocytes (23), this approach has successfully demonstrated the direct involvement of USF.

How USF stimulates transcription remains unclear. It has been shown that USF can overcome transcriptional repression by displacing nucleosomes located at the promoter region (52). It has also been proposed that USF stimulates transcription by interacting with TFIID on the promoter, thus stabilizing the formation of the preinitiation complex (42). USF has also been implicated in transcriptional stimulation through the initiator element (Inr) (9, 25), although the physiological relevance of this function remains to be demonstrated. To gain further insights into the mechanisms by which USF functions as a transcription factor, we undertook a structure-function analysis of the murine USF2 protein to identify the domains involved in nuclear localization and transcriptional activation.

MATERIALS AND METHODS

USF1 and USF2 expression vectors. All expression plasmids for wild-type USF1 and murine USF2 and their deletion mutants were constructed in vector pSG5 (Stratagene). The *Eco*RI-*Nsi*I fragment of the mouse USF2 cDNA (45) and that of the human USF1 cDNA (14) were end filled and subcloned into the *Bam*HI site of pSG5 to generate psvUSF2 and psvUSF1. Construction of the U2ΔB and U2ΔE4 mutants has already been described (27, 29). With the two exceptions described below, the first six amino acids of USF2 were kept in all USF2 deletion mutants to provide the translation start codon. U2Δ(6-40) was generated by digestion of psvUSF2 with *Sma*I and religation. U2Δ(7-123) and U2Δ(7-148) were generated by digestion of psvUSF2 with *Eag*I and the exonuclease III treatment for different lengths of time, followed sequentially by S1 nuclease digestion, end filling by Klenow, *Sma*I digestion, and religation. U2Δ(7-157) was generated by digestion of psvUSF2 with *Eco*RI and *Eag*I, end filling, and religation. U2Δ(7-186) was constructed by inserting the PCR fragment spanning the region between residue 186 and the *Nsi*I site into psvUSF2 cut with *Sma*I and *Bgl*II. U2ΔN and U2Δ(7-239) were generated by digestion of psvUSF2 with *Sma*I and either *Xho*I or *Esp*I, followed by end filling and religation. For construction of U2ΔUSR (internal deletion of residues 208 to 230) and U2ΔUSR2 (deletion of residues 216 to 230), psvUSF2 was digested with *Xho*I,

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treated with *Bal*31 for different lengths of time, end filled by Klenow, and digested with *Eco*RI. The resulting fragments were cloned into psvUSF2 cut with *Eco*RI and *Xho*I. To construct U2HLH-zip, a PCR fragment spanning the USF2 HLH-zip region and part of the 3′ untranslated region upstream of the *Nsi*I site was subcloned into psvUSF2 that had been cut with *Xma*I and *Bgl*II. Mutants U2Δ(1-199) and U2ΔBΔ(1-199) were derived from psvUSF2 and U2ΔB, respectively, by digestion with *Eco*RI and *Eag*I, followed by end filling and religation. The translation start codons in these two mutants are provided by the two consecutive internal methionines located within the USF-specific region (USR). U2ΔBΔUSR was created by subcloning the *Xho*I-to-*Sma*I fragment from U2ΔUSR2 into the *Sma*I-digested U2HLH-zip plasmid. U2ΔE124 was generated by digestion of U2Δ(6-40) with *Eag*I, followed by religation. IB, IUSR, and I(USR+B) were constructed by cloning the PCR fragments spanning residues 200 to 231 (USR), 231 to 249 (BR), and 200 to 249 (USR and BR) into U2ΔBΔUSR digested with either *Xma*I or *Sma*I.

U1Δ(1-130) and U1ΔN were generated by subcloning the *Kpn*I-to-*Xba*I and *Ava*I-to-*Xba*I fragments of psvUSF1 into vector psvUSF2 digested with *Sma*I and *Xba*I. U1Δ(1-157) was generated by subcloning a PCR fragment spanning the sequences between residue 157 of USF1 and the *Xba*I site of psvUSF1 into psvUSF2 cut with *Xma*I and *Xba*I. U1Δ(1-163) was generated by digestion of psvUSF1 with *Eco*RI and *Kpn*I, followed by end filling and religation.

GAL4-USF2 fusions. All GAL4 fusions were constructed in plasmid pSG424 (40), which contains GAL4(1-147) at the N terminus. G-U2N was generated by inserting the end-filled *Bam*HI-to-*Xho*I fragment of psvUSF2 into a pSG424 vector that had been cut with *Bam*HI and *Sac*I and end filled. G-U2(40-231) was generated by digestion of G-U2N with *Sma*I and religation. The fragment between the first *Eag*I site and the *Xho*I site of psvUSF2 (obtained through partial *Eag*I digestion) was end filled and inserted into *Sma*I-digested pSG424 to generate G-U2(96-231). The fragment between the second *Eag*I site and the *Xho*I site of psvUSF2 was similarly cloned to generate G-U2(158-231). G-U2USR was constructed by inserting the *Nco*I-to-*Sac*I fragment of U2Δ(7-186), which spans residues 1 to 6 and 187 to 237 of USF2, into pSG424 cut with *Sma*I and *Sac*I. Point mutations were introduced into the USF2 cDNA at amino acids 199 and 200, which created an *Ase*I site, to generate plasmid pSG5M2-PM. The fragments between the *Bam*HI site and this *Ase*I site or between the first *Eag*I site and the *Ase*I site of this pSG5M2-PM plasmid were subcloned into *Sma*I-digested pSG424 to generate G-U2(9-199) and G-U2(96-199), respectively. G-U2(9-199) was digested with *Eco*RI and *Eag*I and then end filled and religated to generate G-U2(158-199). The fragments between the *Bam*HI site and the first or second *Eag*I site of psvUSF2 were end filled and cloned into the *Bam*HI site of pSG424 to generate G-U2(9-96) and G-U2(9-157), respectively. Finally, G-U2N and G-U2N(9-199) were digested with *Eag*I and religated to generate plasmids G-U2NΔE4 and G-U2NΔE4ΔUSR, respectively. The constructs were verified by DNA sequencing to ensure that all insertions were in the proper frame.

Reporter constructs. Reporter plasmids pU2E1b-CAT, also called pML$_2$E1b-CAT (36), and pG5E1bCAT (28) have been previously described. The pAd2-CAT construct, which contains the natural adenovirus ML promoter sequences from positions −250 to +30 upstream of a chloramphenicol acetyltransferase (CAT) reporter gene, was a generous gift from C. S. H. Young (Columbia University, New York, N.Y.). Plasmids pU2E1b-Inr^{for} and pU2E1b-Inr^{rev} were generated by inserting a double-stranded oligonucleotide containing the sequence 5′ TCTCACTCTCTTCCCCAGCT 3′ into *Sac*I-digested and end-filled plasmid pU2E1b-CAT in the forward and reverse orientations, respectively. The pMLLuc reporter, a generous gift from L. A. Garrett and B. de Crombrugghe (M. D. Anderson Cancer Center, Houston, Tex.), contains the adenovirus ML promoter sequences from positions −46 to +10 inserted between the *Kpn*I and *Hin*dIII sites of promoter-less luciferase reporter plasmid pA3LUC (51). The pU3MLLuc and TATA$^+$Inr$^+$ reporters were generated by inserting in the forward or reverse orientation, respectively, three direct repeats of an oligonucleotide with the sequence 5′ GATCCTTATAGGTGTAGGCCACGTGACCA 3′ into *Sma*I-digested pMLLuc. The TATA$^+$ Inr$^-$ reporter was derived from the TATA$^+$ Inr$^+$ plasmid by changing the 5′ TATAAA 3′ sequence to 5′ TCGAGA 3′ with the Transformer site-directed mutagenesis kit (Clontech) in accordance with the manufacturer's instructions.

Cell culture and transfections. HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and 5% bovine calf serum. Cells were plated 1 day prior to transfection (5×10^5 cells per 10-cm-diameter dish) and transfected with a total of 15 to 20 μg of DNA by the calcium phosphate precipitation method (5). Twelve hours later, the precipitates were washed, and 24 to 30 h after washing, the cells were either harvested for further analysis or fixed for immunostaining.

For CAT assays, the cells were scraped into TEN buffer (40 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl), collected by 30-min of centrifugation at 10,000 rpm in an Eppendorf microcentrifuge, and resuspended in 400 μl of buffer (250 mM Tris-HCl [pH 8]). After five freeze-thaw cycles, the extracts were spun in a microcentrifuge for 5 min at 4°C. CAT assays were performed as previously described (13). For luciferase assays, the cells were resuspended in 100 μl of lysis buffer (25 mM Tris-HCl [pH 7.8], 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, 1% Triton X-100). Luciferase activity was determined by measuring light emission with a scintillation counter by using 100 μl of the assay reagent (Promega) and 5 to 10 μl of extract.

Electrophoretic mobility shift assays and Western blots (immunoblots). Self-complementary oligonucleotide 5′ CTGAATTCCTGGTCACGTGACCGCA GCTGT 3′, which contains the consensus USF binding site, was used as a probe for mobility shift assays after end filling with Klenow and [α-^{32}P]dATP. The DNA-binding reaction mixtures (15 μl) contained 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2.5 μg of poly(dI-dC), 3.3% glycerol, 0.3 ng of probe, and 1.4 μg of protein from whole-cell extract (prepared as for the CAT assay). After 20 min at room temperature, the samples were supplemented with 3 μl of Ficoll and analyzed by electrophoresis on 4% polyacrylamide gels. For Western blot analysis, 7.2 μg of whole-cell extract (as prepared for CAT assays) was applied to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis gels. The proteins were transferred to nitrocellulose filters and probed with USF2 antiserum (46).

Indirect immunostaining. Staining of HeLa cells was carried out essentially as previously described (2). Briefly, transfected HeLa cells were washed three times in phosphate-buffered saline (PBS), fixed for 10 min in freshly prepared 3% paraformaldehyde in PBS, washed three times in PBS, incubated in 0.15% Triton X-100 in PBS, and washed three more times in PBS. The cells were then blocked for 30 to 60 min in a 2% solution of bovine serum albumin in PBS, incubated for 4 h with USF2 antiserum at a dilution of 1:200 in blocking buffer, and then washed three times for 10 min each time in blocking buffer. The dishes were then incubated for 1 h in fluorescein-conjugated goat anti-rabbit immunoglobulin G (Cappel, West Chester, Pa.), which had been diluted 1:1,000 in blocking buffer, and washed three more times before being examined under a fluorescence microscope.

RESULTS

Nuclear localization signals (NLS) of USF2. To ascertain that all deletion mutants to be tested for transcriptional activity would properly localize to the nucleus, it was essential first to identify the regions of USF2 required for nuclear targeting. NLS are usually short stretches of amino acids exposed at the protein surface and containing several characteristic basic amino acids and prolines (1). However, USF2 lacks basic amino acid clusters outside of the basic region (BR) involved in specific DNA recognition (45). To examine the possible involvement of the BR and other sequences of USF2 in nuclear localization, various deletion mutants were generated and used for transient transfection of HeLa cells. The subcellular distribution of these proteins was then examined by indirect immunofluorescence staining of the transfected cells (Fig. 1).

As anticipated from the suspected role of USF2 in DNA transcription, wild-type USF2 localized entirely to the nucleus, with the nucleolar regions clearly excluded (Fig. 1E). Progressive deletions in the N-terminal region of USF2 did not affect nuclear localization, so long as the BR-HLH-zip domain remained intact. However, some cytoplasmic staining was observed when the deletion extended into a portion of the BR, and removal of the entire BR abolished preferential nuclear localization (Fig. 1A and E). These results strongly suggested involvement of the BR in the nuclear localization of USF2. In agreement with this observation, the amino acid sequence of the USF2 BR (Fig. 1D) displays homology to some of the split NLS previously identified in other proteins (8). Further investigations revealed, however, that the BR is not the only domain capable of targeting USF2 to the nucleus. Indeed, an internal-deletion mutant lacking most of the BR (U2ΔB) still appeared entirely in the nucleus (Fig. 1B and E). By introducing additional mutations within the context of this BR deletion, we narrowed the location of a second putative NLS to a 29-amino-acid stretch within the USR, a highly conserved region of USF normally located just upstream of the BR (Fig. 1B and E). This result was quite surprising because the amino acid sequences of the USR apparently involved in nuclear targeting bear no obvious resemblance to classical NLS (Fig. 1D).

Internal deletion of both the BR and USR of USF2 abolished preferential nuclear staining (Fig. 1B), suggesting that these were the only two regions of USF2 involved in nuclear localization. However, one could still argue that the inability of

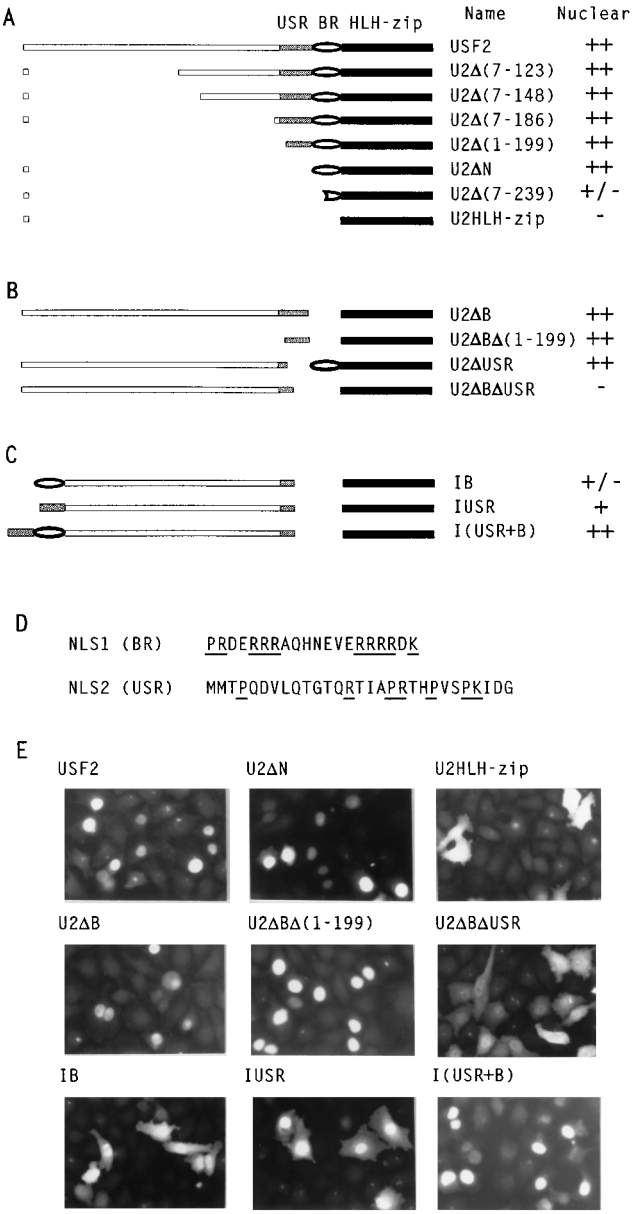

FIG. 1. Domains involved in the nuclear localization of USF2. Various deletion mutants of USF2 were transiently expressed in HeLa cells, and their subcellular distribution was examined by indirect immunofluorescence analysis with USF2-specific antibodies. (A) The basic region of USF2 can function as an NLS. Schematic representations of the various USF2 mutants are shown at the left, and the locations of functional domains are indicated at the top. The subcellular localization observed for each protein is indicated at the right as follows: ++, exclusively nuclear; +, mostly nuclear; +/−, significant cytoplasmic staining; −, equally distributed between the cytoplasm and the nucleus. (B) The USR of USF2 can function as an NLS. (C) Outside of their normal context, the BR and USR of USF2 are both required to trigger complete nuclear localization. (D) Amino acid sequences of the two regions involved in the nuclear localization of USF2. The prolines and basic residues often found in NLS are underlined. (E) Examples illustrating the subcellular distribution of various USF2 mutants. Transfected cells stained with USF2 antibodies and fluorescein-conjugated secondary antibodies were photographed under a fluorescence microscope (magnification, ×83).

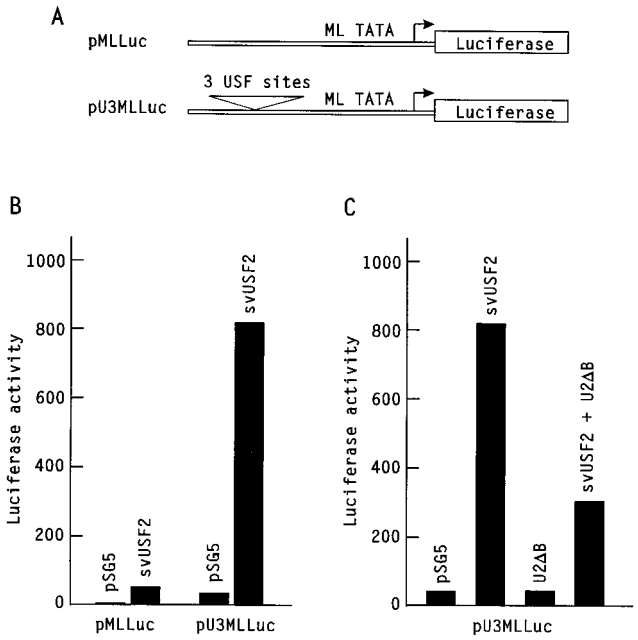

FIG. 2. Transcriptional activation by USF2 requires DNA binding. (A) Schematic representation of the two luciferase reporters. Both plasmids contain the adenovirus ML minimum promoter (positions −46 to +10) with or without upstream insertion of three tandem repeats of the ML USF specific binding site. (B) Specific binding sites are required for effective transcriptional activation by USF2. HeLa cells were cotransfected with 10 μg of pMLLuc or pU3MLLuc and 6 μg of either the parental pSG5 vector or USF2 expression plasmid psvUSF2, as indicated. The total amount of DNA in each transfection was kept at 20 μg by addition of pSG5. The cells were harvested 40 h after transfection, and the cellular lysates were assayed for luciferase activity. (C) DNA binding-dependent transcriptional activation by USF2. The U2ΔB mutant, which is defective in DNA binding because of internal deletion of the BR (see Fig. 3C), was tested in the cotransfection assay for transcriptional activity and a dominant-negative effect over wild-type USF2. Cotransfection was carried out with 10 μg of pU3MLLuc and 6 μg of the expression vector indicated above each bar. Luciferase activities were determined 40 h after transfection.

certain mutants to localize to the nucleus was the result of conformational changes that masked the real NLS. To eliminate this possibility and confirm the existence of NLS in the BR and USR, several additional mutants were generated (Fig. 1C). These contained the USR or BR of USF2 inserted, separately or together, at the N terminus of nuclear localization-deficient mutant U2Δ(USR+B). Surprisingly, the BR alone in this new location did not direct efficient nuclear localization. Thus, unlike classical NLS, the BR of USF2 requires a particular conformation or context for its nuclear localization function. In contrast, addition of the USR alone at the N terminus of U2Δ(USR+B) clearly resulted in preferential nuclear staining, although there was still some residual staining in the cytoplasm. Insertion of both the USR and BR resulted in complete nuclear localization, confirming the importance of these two regions in the nuclear targeting of USF2 (Fig. 1C and E).

Transcriptional activation by USF2 requires DNA binding. The transcriptional activity of USF2 in vivo and its dependence upon specific DNA binding were first investigated by carrying out transient cotransfection assays with HeLa cells (Fig. 2). The luciferase reporter plasmids used in these experiments contained the adenovirus ML TATA box and Inr elements with (pU3ML-Luc) or without (pML-Luc) the upstream insertion of three tandem repeats of a USF-specific binding site (Fig. 2A). As shown in Fig. 2B, cotransfection of wild-type USF2 with the pU3ML-Luc reporter resulted in a 20- to 30-fold increase in luciferase activity compared with transfections carried out with the empty expression vector. This result indicated that like USF1 (9), USF2 is a transcriptional activator in vivo. Note, however, that as reported for USF1 (9), USF2

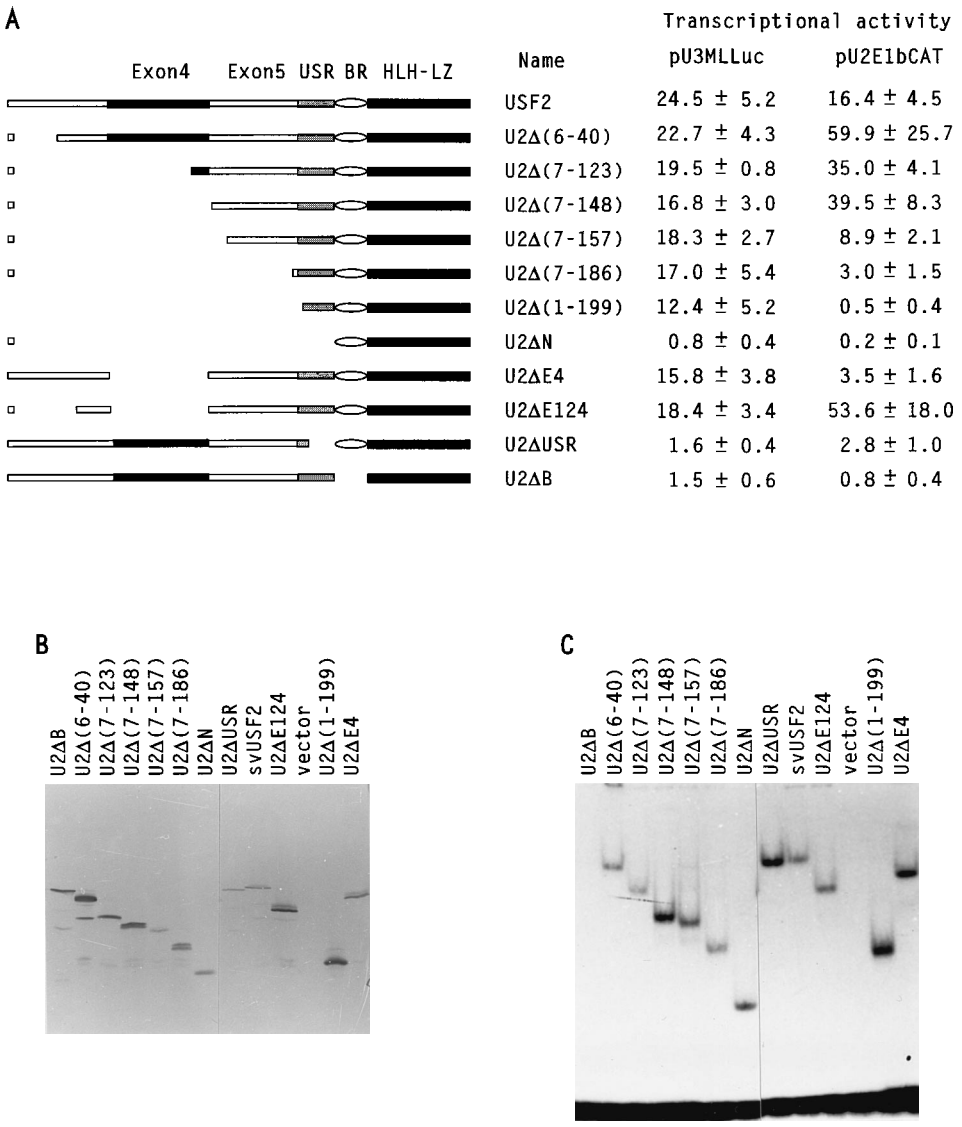

FIG. 3. Transcriptional activities of various deletion mutants of USF2. (A) Schematic representations of the USF2 deletion mutants are shown at the left, and the various functional domains are indicated at the top. Transfections were carried out as described in the legend to Fig. 2, except that two different reporter constructs were utilized. The values shown at the right for each mutant are the averages and standard deviations calculated from 3 to 10 independent experiments carried out with different plasmid preparations. In some of the experiments, the two reporters were cotransfected to minimize variations. The results shown are ratios of the luciferase or CAT activities observed in the presence of the various proteins to the levels observed in the absence of USF. The levels of expression of the various USF2 mutants were verified by Western blot analysis (B) and electrophoretic mobility shift assay (C).

overexpression also activated transcription from plasmid pML-Luc lacking specific USF sites. However, the overall luciferase activity was, in this case, 8- to 10-fold lower than when specific USF binding sites were present (Fig. 2B). In the case of USF1, this binding site-independent activation was attributed to stimulation by USF through the Inr element. Although such a mechanism might also contribute to the stimulation of pML-Luc by USF2 (see below), it is also possible that the overexpressed USF stimulates transcription in the absence of specific binding sites by interacting with cryptic USF-like sites in various regions of the reporter plasmid.

To verify that transcription stimulation by USF2 required, in all cases, interaction of the transcription factor with the DNA, we examined the effect of the BR deletion present in the U2ΔB construct. Expression of U2ΔB had no effect on the activity of the ML promoter, regardless of whether USF-binding sites were present (Fig. 2C) in the reporter construct or not (data not shown). However, cotransfection of U2ΔB significantly decreased the stimulation observed with wild-type USF2, demonstrating that the BR deletion did not abolish the dimerization ability of USF2 and that interaction with the promoter DNA is essential for transcriptional activation by USF2.

The USR is a transcriptional activation domain. To delineate domains in USF2 specifically required for transcriptional activation, we first used the above-described cotransfection assay with the pU3ML-Luc reporter construct (Fig. 3). Since the C-terminal BR-HLH-zip domain of USF2 is necessary and sufficient for both dimerization and DNA binding (45, 46), all deletion mutants (with the exception of U2ΔB) were designed to keep this domain intact, thus ensuring equal DNA binding and complete nuclear localization. Western blot analysis (Fig. 3B) and electrophoretic mobility shift assays (Fig. 3C) with

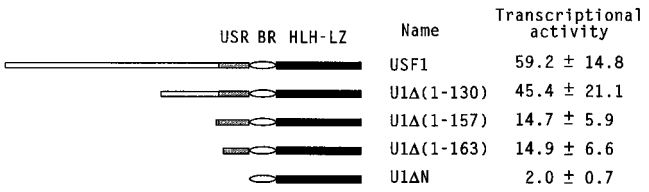

FIG. 4. The USR is also a transcription activation domain in USF1. HeLa cells were cotransfected with the pU3MLLuc reporter and expression vectors for the various deletion mutants of human USF1 schematically represented at the left. The luciferase activities shown are relative to the activity observed with the vector alone. The values are averages from three independent experiments.

transfected cell extracts confirmed that all of the mutants generated were expressed at similar levels and displayed comparable DNA-binding activities. As shown in Fig. 3A, deletions that removed only sequences encoded by the first five exons of the USF2 gene minimally affected transcriptional activation of the pU3ML-Luc reporter gene. For example, deletion mutant U2Δ(7-186), in which only the USR was left intact, displayed 70% of the wild-type USF2 activity. Even mutant U2Δ(1-199), which contained only the last 30 amino acids of the USR, still retained 50% of the wild-type activity. In contrast, the U2ΔN mutant, composed only of the BR-HLH-zip domain, was totally inactive. These results indicated that the USR is necessary and sufficient for transcriptional activation of the adenovirus ML promoter by USF2. In agreement with this observation, an internal deletion that removed just 20 amino acids of the USR (construct U2ΔUSR, Fig. 3A) abolished transcription activation by USF2.

The USR is highly conserved in all USF proteins isolated so far. There is, for instance, 80% homology between the USF1 and USF2 proteins in this region. Such homology suggests that the USR could also play a role in transcription stimulation by USF1. To investigate this possibility, we constructed vectors expressing either full-length USF1 or several N-terminal deletion mutants of USF1. These constructs were tested for DNA binding (data not shown) and subsequently assayed for transcriptional activation of the pU3ML-Luc reporter (Fig. 4). As was observed with USF2, the C-terminal DNA-binding domain of USF1 was insufficient for transcriptional activation. However, when the USR or most of the USR was present, substantial transcriptional activity occurred. Although the activity of these particular mutants was only 20 to 30% of that of the wild-type USF1 protein, this result strongly suggests that the conserved USR has a key role in transcriptional stimulation by all USF proteins.

Mapping of the activation domain of USF2 through a heterologous DNA-binding domain. A strategy commonly used to map the activation domain of a transcription factor is to engineer fusion proteins between a heterologous DNA-binding domain and different parts of the protein of interest and determine the transcriptional activity of the resulting hybrids on an appropriate reporter gene. We used this approach as an independent method to assess the presence of activation domains in USF2. Various segments of USF2 were fused to the GAL4 DNA-binding domain by using the GAL4(1-147) portion, which is known to be sufficient for dimerization, DNA binding, and nuclear localization but does not, by itself, activate transcription (19). The C-terminal BR-HLH-zip domain of USF2 was excluded from these fusions to avoid possible interference with the DNA binding of GAL4. Expression vectors for the GAL4-USF2 chimeras were cotransfected into HeLa cells with a CAT reporter gene driven by a minimal E1b promoter with five upstream GAL4 binding sites. For each hybrid, the level of expression was determined by Western blotting (data not shown) and transcriptional activity was measured by CAT assays. The results of these experiments are summarized in Fig. 5. Fusion of GAL4(1-147) with the entire N-terminal region of USF2 (residues 6 to 231) resulted in a hybrid protein that stimulated transcription from the reporter gene 30- to 35-fold over that obtained with GAL4(1-147) alone. This result confirmed the presence of an activation domain in USF2. Surprisingly, however, removal of the USR [construct G-U2(9-199)] enhanced activity and a fusion containing only the USR (G-U2USR) displayed no activity at all. Instead, comparison of the results obtained with the different constructs revealed that the domain of USF2 responsible for transcriptional activation in this assay corresponded to the exon 5-encoded sequences. Indeed, only the fusions containing the exon 5 region showed significant levels of activation and all fusions lacking it were essentially inactive. Furthermore, construct G-U2(158-199), which contained only the exon 5 region, displayed significant activity. Note, however, that enhanced activity was reproducibly observed when neutral sequences were present between the GAL4 DNA-binding domain and the exon 5 activation domain. In all likelihood, such insertions favor activation by providing conformational flexibility (34, 44).

Activities of USF2 mutants with different reporter constructs. Of the two different assays used to map the activation domain of USF2, one pointed to the importance of the USR, while the other pointed instead to the exon 5 region. Besides the obvious structural difference in the proteins used in these two sets of assays, a possible cause for this apparent discrepancy was the difference in structure between the two reporter constructs used. The E1b TATA box, five upstream binding sites, and a CAT reporter gene were used to analyze the GAL4-USF2 fusions, while the minimum ML promoter with three upstream USF-binding sites and a luciferase reporter gene were used to analyze the internal-deletion mutants of USF2. To determine the influence of these parameters on transcriptional activation by USF2, we retested the activities of several USF2 mutants by using different reporter constructs (Fig. 6). Results essentially identical to those originally obtained with pU3MLLuc were obtained with a CAT reporter gene driven by the natural ML promoter sequences which

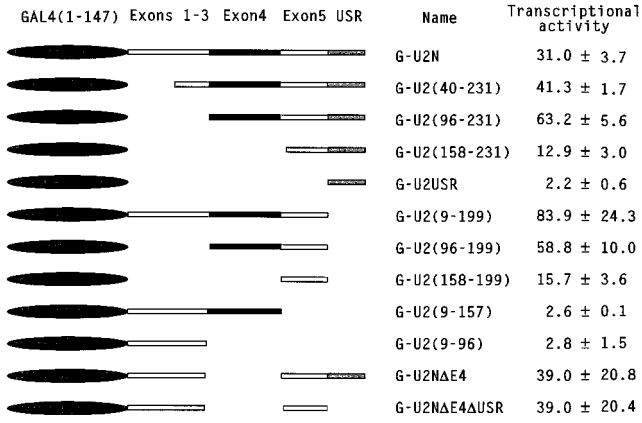

FIG. 5. Mapping of the activation domain of USF2 through a heterologous DNA-binding domain. The transcriptional activities of different GAL4-USF2 fusion proteins (8 μg of each plasmid) were determined by cotransfection with pG5E1bCAT, a CAT reporter construct composed of five GAL4 binding sites upstream of the E1b TATA box (10 μg per transfection). The CAT activity results shown are averages from three different experiments, and the values are normalized against the activity observed with GAL(1-147) alone.

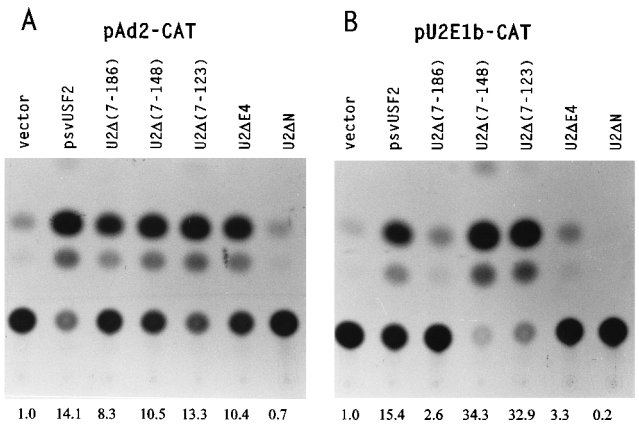

FIG. 6. Transcriptional stimulation by USF2 on different reporter constructs. The transcriptional activities of wild-type USF2 and several representative deletion mutants of USF2 were determined by using two different reporter genes. HeLa cells were cotransfected with 6 μg of each expression plasmid and 10 μg of the pAd2-CAT reporter (A), which contains the natural ML promoter sequences from −250 to +33 driving CAT, or plasmid pU2E1b-CAT (B). The autoradiograms from a representative experiment are shown, and the quantitation indicated below each lane is the ratio of the CAT activity observed in each case to the activity observed with the parental pSG5 vector.

contains a single USF-binding site (Fig. 6A). Again, the USR seemed both necessary and sufficient for full transcriptional activity. This observation demonstrated that the nature of the reporter gene and the number of upstream USF-binding sites are not critical parameters. In contrast, entirely different results were obtained when the same mutants were analyzed with a reporter gene driven by the E1b TATA box (Fig. 6B). For instance, the U2Δ(7-186) and U2ΔE4 mutants, which were fully active with the ML reporter, displayed very little activity in this new context. It appeared, therefore, that the nature of the minimum promoter present in the reporter construct was a key element in determining the activities of various USF2 mutants.

A more complete comparison of the relative activities of USF2 mutants on the E1b and ML reporters is shown in Fig. 3. Binding of wild-type USF2 upstream of the E1b TATA box stimulated transcription 15- to 20-fold, while the U2ΔN mutant, which contained only the BR-HLH-zip domain, repressed endogenous transcription levels very noticeably. This repression was presumably due to the occupancy of the USF sites by this transcriptionally inactive mutant, which probably interferes with endogenous USF activity. In contrast to the results obtained with the ML promoter, mutants containing only the USR were essentially inactive on the E1b reporter. However, most constructs that contained both the USR and the exon 5 region displayed high activity. This observation indicated that the exon 5 region could also function as an activation domain within its normal context in USF2, just as in the context of the GAL4 fusions. Within USF2, however, the overall activity of the exon 5 activation domain seemed to be susceptible to both positive and negative effects of surrounding protein regions. For instance, internal deletion of the USR abolished transcriptional activity, suggesting that the USR is, directly or indirectly, required for the activity of the exon 5 activation domain in its natural context. Possibly, the USR simply provides the appropriate spacing between the DNA-binding domain of USF2 and the exon 5 activation domain. Perhaps more unexpected were the effects observed upon deletion of other USF2 regions. When the sequences corresponding to exons 1 and 2 were deleted from wild-type USF2, enhanced transcriptional activity was observed. This result suggests that these N-terminal sequences can inhibit the activity of USF2. Consistent with this possibility, this region is rich in alanine residues, which is common for transcriptional repressors (15, 26). On the other hand, the USF2 mutant lacking only exon 4 was, by itself, a very weak activator. However, removal of exons 1 and 2 from the exon 4 deletion mutant restored transcriptional activity (construct U2ΔE124), which indicates that the exon 4 sequences modulate the negative effect produced by the most N-terminal exons. Note that this phenomenon may well have physiological relevance because USF2 lacking exon 4 is a naturally occurring isoform of USF that is generated by alternative splicing mechanisms (27).

Role of Inr in transcriptional activation by USF2. One major difference between the E1b and ML minimum promoters is that the latter contains an Inr in addition to the TATA box (47). Since the TATA and Inr elements are the only known elements of minimal promoters (53), it seemed possible that the lack of an Inr in the E1b promoter was responsible for its differential response to some of the USF2 mutants. To investigate this possibility, we modified the pU2E1b-CAT reporter by inserting, in the forward or reverse orientation, the ML Inr element downstream of the E1b TATA box (Fig. 7A). These modified reporters (pU2E1b-Inr^{for} and pU2E1b-Inr^{rev}) were then compared to the original construct in a cotransfection

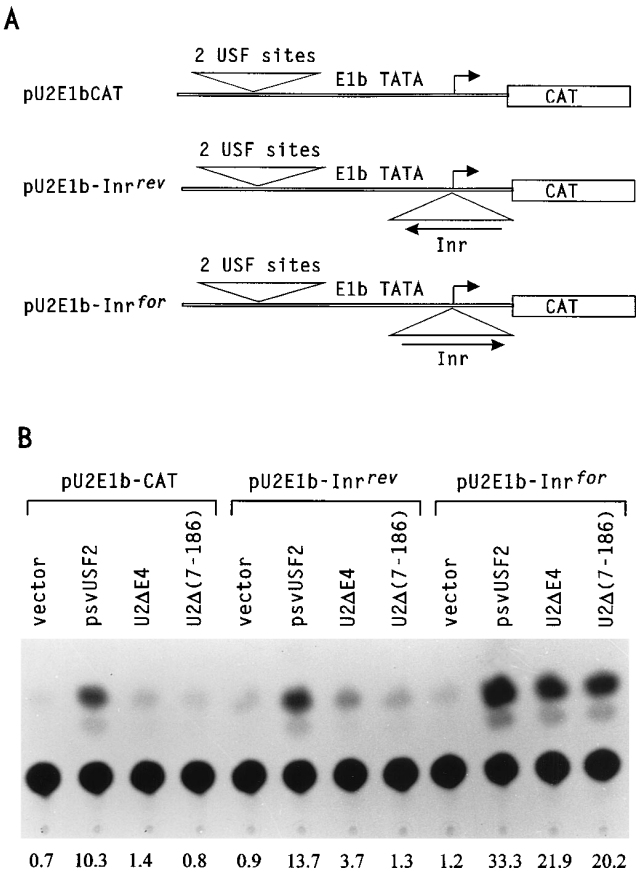

FIG. 7. Transcriptional activities of USF2 mutants in the presence or absence of an Inr element. (A) Schematic representation of the three different reporter constructs. (B) Transcriptional activities of the wild-type USF2 and two representative deletion mutants on the different reporters. HeLa cells were transiently transfected with 6 μg of each of the indicated expression plasmids and 10 μg of the indicated reporter plasmids as described in the legend to Fig. 2. Quantitation of CAT activities is shown under each lane as conversion rates.

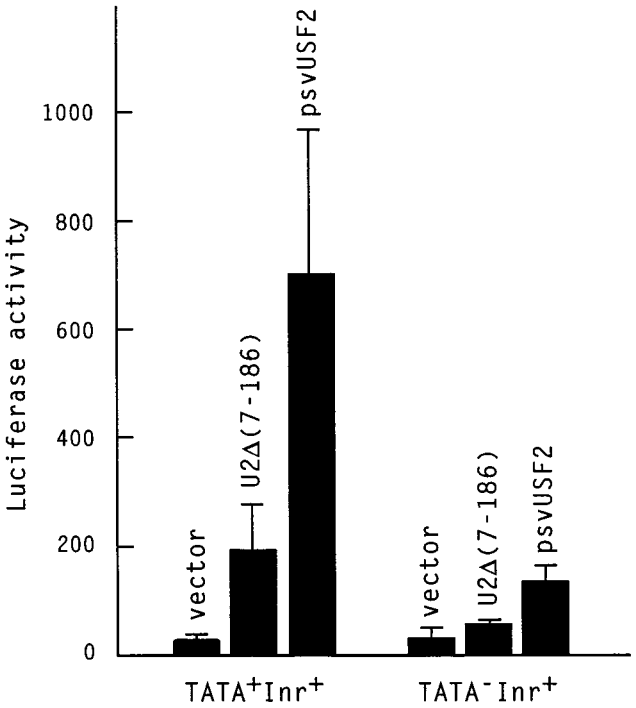

FIG. 8. Maximal activation by USF2 requires the presence of a TATA box, even in the presence of an Inr element. The two reporters used in this experiment were identical to three inverted copies of the ML USF binding site and the ML Inr element upstream of a luciferase gene, except that the ML TATA box sequence (TATAAA) present in the TATA$^+$ Inr$^+$ reporter was mutated to a TCGAGA sequence in the TATA$^-$ Inr$^+$ reporter. Transfections were carried out as described for Fig. 2 and 3.

assay with the wild-type USF2 and the two mutants, U2Δ(7-186) and U2ΔE4, whose activity was most dependent upon the promoter context. As illustrated in Fig. 7B, all three reporters were activated by wild-type USF2, although the response of pU2E1b-Inr^{for} was nearly threefold that of the other reporters. However, the activity of the two USF2 mutants was drastically affected by the presence of the Inr. Both U2Δ(7-186) and U2ΔE4 stimulated pU2E1b-Inr^{for} 15- to 20-fold, while these mutants were essentially inactive in the absence of the Inr or when the element was present in the reverse orientation. In contrast, the U2ΔN mutant, lacking all known activation domains, remained entirely inactive on all three reporters (data not shown). Taken together, these results suggest that the Inr element can play a key role in the activation of various genes by the transcription factor USF. In particular, since Inr-dependent activation was observed with the U2Δ(7-186) mutant that contains only the USR, the presence of an Inr element seems essential for the activity of the USR activation domain.

To investigate whether the TATA box is also required for activation of Inr-containing promoters by USF2, we compared the activities of both the wild-type USF2 and U2Δ(7-186) proteins on two reporter constructs that differed only by mutation of the normal ML TATA box sequence 5′ TATAAA 3′ (TATA$^+$ Inr$^+$ plasmid) to a 5′ TCGAGA 3′ sequence (TATA$^-$ Inr$^+$ plasmid) (Fig. 8). This experiment revealed that, at least in this artificial promoter context, the TATA box is essential for efficient activation by USF2. Indeed, disruption of this element decreased the effect observed with wild-type USF2 from 24-fold to only 4-fold stimulation while activation by the U2Δ(7-186) mutant decreased from the original 7-fold to less than 2-fold stimulation. Note that the somewhat reduced activity of the U2Δ(7-186) mutant relative to the wild-type USF2 protein in this experiment seems to correlate with the fact that the ML USF sites in the TATA$^+$ Inr$^+$ reporter are located farther away from the initiation site and in the opposite orientation compared with the natural ML promoter or the pU3MLLuc reporters. This observation indicates that, in contrast to the mutants, the different activation domains present in the wild-type USF2 protein allow more flexibility in the variety of promoter constructs that it can efficiently activate.

Taken together, our results obtained with different minimum promoters suggest that the presence of an Inr element, although essential for the activity of the USR activation domain, may be, by itself, insufficient to trigger a response of the transcriptional apparatus to proximally bound USF2 proteins. However, because the promoters used in these experiments were artificial, the possibility exists that other structural elements, or the actual promoter configuration of natural TATA-less promoters, could, under other circumstances, permit efficient stimulation by USF, even in the absence of a TATA box.

DISCUSSION

The studies reported here extend our understanding of the structure-function relationship of USF2 to include most of the nonconserved N-terminal regions (Fig. 9). Determination of the genomic structure of the USF2 gene had previously shown a correlation between discrete functional domains of this transcription factor and their distribution in separate exons (27). For instance, the highly conserved USR, originally defined by amino acid sequence comparisons, corresponds precisely to USF2 exon 6, while the BR is encoded by exon 7. The identification here of a separate transcriptional function for the exon 5 sequences, as well as of a regulatory role for the alternate exon 4 domain, further extends this correlation between the structure of the USF2 gene and the various functional domains of this transcription factor (Fig. 9).

We identified here two distinct regions, one within the USR and one in the BR, that seem to be implicated in the nuclear localization of USF2. In its natural location, each of these regions could independently mediate complete nuclear localization. Why, then, the redundancy? It could be that, for steric reasons, one of these two NLS is usually nonfunctional. For instance, we found that the BR, when mislocated, could not, by itself, direct nuclear localization, which suggests that the folded structure of USF2 plays an important role in nuclear translocation. Another possibility is that interaction with some cellular proteins at times masks one of the two NLS of USF2. Alternatively, the two signals may simply function, either additively or cooperatively, to ensure maximum nuclear localization. In support of this idea, the combined USR and BR signals mediated complete nuclear localization even when placed out of their natural context, while each region alone was, in this case, insufficient. It is noteworthy that transcription factor c-

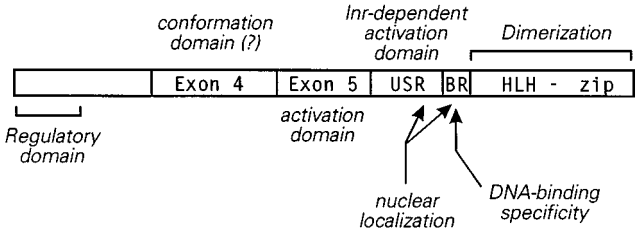

FIG. 9. Schematic representation of transcription factor USF2 indicating the locations of the various functional domains.

Myc, which is structurally very similar to USF, also has two NLS, one upstream of the BR and the other within the BR itself. In the case of Myc, however, the BR signal was shown to be more potent than the upstream signal (6).

Our mutational studies have identified two transcriptional activation domains in USF2, one in the exon 5-encoded domain and the other in the USR (exon 6). Because these two regions are adjacent in the wild-type USF2 protein (Fig. 9), one could also consider them two parts of a larger activation domain with separate, although complementary, functions. The amino acid sequences of the USF2 exon 5 region and the USR bear no obvious resemblance to those of easily identifiable activation domains that are often enriched in particular amino acids (e.g., glutamine-rich, proline-rich, or acidic activation domains; reviewed in reference 30). However, the USR contains a relatively high percentage of serine and threonine residues (5 of 35), including potential phosphorylation sites for caseine kinase II and mitogen-activated protein kinase (12, 20). This indicates that phosphorylation could play a role in regulating the activity of USF.

Of the two activation domains of USF2, only the USR is clearly present in all of the known USF proteins. In fact, this region in human, *Xenopus*, and sea urchin USFs contains almost as many unchanged residues as the HLH domain itself (46). This high degree of evolutionary conservation strongly suggests that the USR plays the same key role in all of these proteins. This possibility is further supported by our demonstration that the USR of USF1 is also capable of activating transcription through the ML promoter. Given the dual role of the USR in both transcriptional activation and nuclear targeting, its relatively small size, and its immediate proximity to the DNA-binding domain, the existence of strong constraints on the evolution of this region becomes quite understandable. In contrast, the exon 5 activation domain of USF2, like the internal activation domain earlier mapped in human USF1 (21), is located in a highly divergent region. This divergence suggests that the USF1 and USF2 proteins are separately regulated and regulate different sets of cellular genes.

One of the most interesting features of the USF2 activation domains is that they are so strongly dependent upon their context, with regard both to the required promoter elements and the surrounding protein domains. The prevalence of such context-dependent activation domains is unclear, because few investigations of transcriptional activation have, as in this case, utilized different approaches (e.g., fusion with different DNA-binding domains) and also different reporter constructs. Our first two sets of deletion mutants, engineered by using either the USF2- or the GAL4 DNA-binding domain, identified, respectively, the USR and exon 5 activation domains as both necessary and sufficient for transcriptional activity. These two conclusions seemed contradictory, since the USR failed to show any activity in the context of GAL4 fusions, while exon 5 was inactive in the second assay system. This apparent contradiction was essentially resolved by our finding that different minimum promoters discriminate between the two activation domains. It is very clear from our data that the USR specifically activates promoters that contain an Inr. However, with or without an Inr, we could not demonstrate any transcriptional activity of the USR in the context of a heterologous DNA-binding domain such as that of GAL4, and in fact, the fusion proteins containing this domain were always less active than those lacking it (data not shown). It may be that, as shown for the activation domain of the rat glucocorticoid receptor (24), a specific conformation is required for the specialized function of the USR, which is induced only following interaction of the adjacent BR with the DNA (11). Alternatively, activation by USF through the Inr element may involve not only the USR but also residues located within the C-terminal DNA-binding and dimerization domain.

In the absence of an Inr, wild-type USF2 can still stimulate transcription because of the additional activation domain in exon 5. The activity of this exon 5 domain, although detectable in the context of different DNA-binding domains, was also particularly sensitive to the surrounding protein sequences. In the GAL4 fusions, for instance, intervening sequences between exon 5 and the DNA-binding domain enhanced its activity, while the USR moderately inhibited it. Within USF2 itself, the N-terminal sequences, exon 4, and the USR strongly influenced the overall transcriptional activity of the exon 5 activation domain. These effects probably reflect different conformations adopted by the various mutant proteins, raising the intriguing possibility that in the cell, conformational changes brought about by interactions with other proteins could similarly modulate the *trans*-activating potential of this transcription factor.

An influence of core promoter elements on transcriptional activation has been observed in several cases (7, 48), but most activation domains were found to stimulate equally well TATA box- and Inr-dependent transcription (4). In contrast, the very low activity demonstrated even by the wild-type USF2 protein on a reporter plasmid containing an Inr element but lacking a TATA box (Fig. 8) indicates that the basic promoter context can be extremely important for transactivation by USF2.

The USR is the first example of a discrete activation domain whose function is entirely dependent on the presence of an Inr. Given the current understanding of the proteins and mechanisms involved in basic and activated transcription, one can only speculate on how the USR may stimulate transcription in the presence of an Inr but not in its absence. There are reports indicating that different transcription factors, including USF itself, may directly interact with the Inr (39). However, it is clear that this element is also directly recognized by the TFIID complex (17, 37, 49, 50). Interaction with the Inr correlates with an altered conformation of the TFIID-DNA complexes, which is clearly observed in DNase I footprinting (33, 50). Whether this different conformation of TFIID on promoters containing or lacking an Inr is accompanied by variations in the polypeptide composition of the resulting preinitiation complex remains to be determined. In either case, this difference in composition or conformation of the preinitiation complex brought about by the Inr is probably essential to trigger direct or indirect interaction of the USR activation domain with the basic transcriptional apparatus.

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