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Cooperative interactions between transcription factors Sp1 and OTF-1

(enhancer/transcriptional activation/U2 RNA gene)

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ABSTRACT **We have examined whether the functional synergism between transcription factors Sp1 and OTF-1 involves cooperativity in binding. To demonstrate cooperativity, synthetic enhancers were constructed in which Sp1-binding sites were combined with various OTF-1-binding sites that differed in their binding affinities. The ability of these constructions to activate transcription from the human U2 small nuclear RNA promoter was measured. The results showed that an Sp1-binding site stimulated transcription 2-fold when combined with a high-affinity binding site for OTF-1. When combined with a low-affinity OTF-1-binding site, in contrast, a 20-fold stimulation of transcription was observed. The stimulatory effect of Sp1 was moreover influenced by the distance between the Sp1- and OTF-1-binding sites and the functional cooperation was mirrored by the cooperative formation of OTF-1- and Sp1-specific protein–DNA complexes *in vitro*. We conclude from these results that the functional cooperation between OTF-1 and Sp1 involves physical interactions between the two transcription factors resulting in cooperative binding. The results thus reveal a mechanism by which Sp1 can modulate transcription.**

Studies of functional and structural interactions between mammalian transcription factors have been hampered by the complexity of most promoter/enhancer elements and by their functional redundancy (1–3). In the present communication, we have studied the possible interactions between the transcription factors that bind to the enhancer element of a human U2 small nuclear RNA (snRNA) gene. U2 snRNA genes are transcribed by RNA polymerase II (4, 5), and the transcription is dependent on a distal enhancer that functions in conjunction with a proximal sequence element (PSE), located 50–60 base pairs (bp) upstream of the cap site (6–10). It has been shown (11, 12) that the U2 enhancer includes one so called octamer motif ATGCAAAT (13), which is the binding site for the transcription factor OTF-1 in nonlymphoid cells (14), and three binding sites for the ubiquitous transcription factor Sp1 (15, 16). A previous characterization of the U2 enhancer revealed a nonadditive functional cooperation between the OTF-1- and the adjacent Sp1-binding sites (17). The smallest functional unit capable of supporting wild-type transcriptional levels consisted of one OTF-1-binding site with an adjacent Sp1-binding site. The structural simplicity of this enhancer element makes it suitable for studies of how transcription factors cooperate to activate transcription. In the present communication, we present results from which we conclude that the functional cooperation between the transcription factors Sp1 and OTF-1 involves physical interactions that result in cooperative binding to their juxtaposed sequence motives.

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MATERIALS AND METHODS

Generation of U2M: Constructions. The construction of the U2M:Sp1,Octa and the U2M:−Enh enhancer mutations has been described (17). Additional U2M: constructions were generated in the same way as U2M:Sp1,Octa by cloning the double-stranded oligodeoxyribonucleotides, specified in Figs. 2 and 4, upstream of a marked U2M maxigene lacking an enhancer element.

Transfections and S1 Nuclease Mapping. Transfections into HeLa cells followed by S1 nuclease analysis of marked U2M maxigene transcripts were performed as described (17). Variations in transfection efficiencies were adjusted by cotransfecting 4 μg of the pSXβ+ reference plasmid (18), with the exception for the transfections shown in Fig. 3*A*. The calculation of the transcription efficiencies was based on an average of at least four transfections using two plasmid preparations with standard deviations of ≈10%.

Gel Mobility Shift Analysis. The double-stranded oligodeoxyribonucleotides, used for the construction of plasmids U2M:A, U2M:C, and U2M:C+15 (see Figs. 2 and 4), were used as [α-^{32}P]dCTP 3′ end-labeled probes. The double-stranded oligodeoxyribonucleotides used for the U2M:Octa, U2M:3xSp1, and U2M:NFI constructions (Figs. 1 and 3; ref. 17) were used as OTF-1-, Sp1-, and NFI-specific competitors, respectively. The reactions were performed as follows: an 18-μl preincubation reaction mixture containing the specified amount of sequence-specific competitor DNA, 4 μg of poly(dI-dC)·poly(dI-dC), 4 μl of a crude nuclear extract (8 mg/ml), 12% (vol/vol) glycerol, 12 mM Hepes (pH 7.9), 60 mM KCl, 0.12 mM EDTA, 0.30 mM dithiothreitol, and 0.30 mM $ZnCl_2$ was incubated for 15 min at 37°C. The molar concentrations were calculated on a final volume of 20 μl. The nuclear extract was prepared as described by Dignam *et al.* (19), modified by the inclusion of 0.5 mM $ZnCl_2$ in the dialysis buffer, which was found to favor Sp1 interactions without affecting the OTF-1 interactions. The reaction mixtures were incubated for another 15 min at 37°C after the addition of 2 μl (0.25 ng) of ^{32}P-labeled probe. This incubation was followed by a 1:5 dilution of the reaction mixture in a buffer containing 12% glycerol, 12 mM Hepes (pH 7.9), 60 mM KCl, 0.12 mM EDTA, 0.30 mM dithiothreitol, and 0.30 mM $ZnCl_2$. The samples were incubated for another 15 min at 37°C before loading 20% (20 μl) of the reaction mixture onto a nondenaturing 4% polyacrylamide gel [acrylamide/*N*,*N*′-methylenebisacrylamide, 30:1 (wt/vol)] containing 50 mM Tris base and 380 mM glycine. Autoradiographic films were scanned densitometrically. The relative amount of radioactive probe found in the various complexes (C1a, C1b, or C2) or as unbound probe (F) was estimated by calculating the fraction of probe in one band as compared with the sum of the bands (F, C1a, C1b, and C2) in a given lane.

Abbreviations: snRNA, small nuclear RNA; PSE, proximal sequence element.

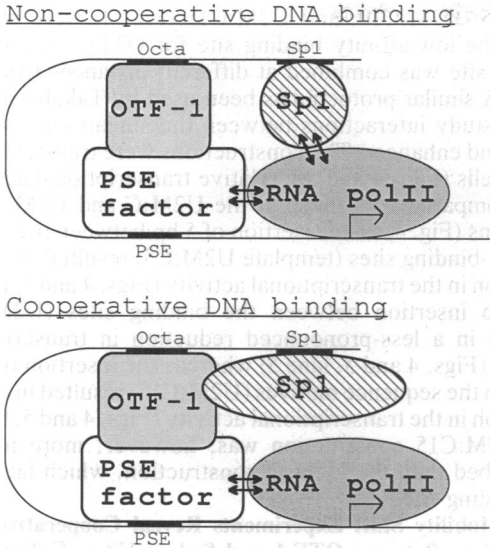

FIG. 1. Two models that could account for the functional cooperation between Sp1 and OTF-1 in the U2 snRNA gene enhancer. Relatively stable interactions are indicated by overlaps, whereas the arrows indicate weaker transient interactions. A possible stable interaction between OTF-1 and a putative DNA-binding factor interacting with the PSE is included in the models to illustrate the importance of OTF-1 for efficient transcription. pol II, polymerase II. (*Upper*) Model A. (*Lower*) Model B.

RESULTS

The Stimulatory Effect of a Low-Affinity Binding Site for OTF-1 Increases Drastically When It Is Combined with an Sp1-Binding Site. The previously reported functional cooperation between Sp1 and OTF-1 in a U2 snRNA gene enhancer (17) could be explained by two principally different models, which are depicted in Fig. 1. According to model A, the transcription factors Sp1 and OTF-1 interact with the DNA template independently (the noncooperative DNA-binding model). Model B, in contrast, predicts that the two proteins bind to the DNA template cooperatively (the cooperative DNA-binding model). In this model the role of Sp1 would be to facilitate the binding of OTF-1 to its cognate motif with or without making additional contacts with other components of the transcriptional machinery.

If physical interactions do occur between the two transcription factors as in model B, it would be expected that an Sp1-binding site would have a relatively stronger stimulatory effect on transcription if it was combined with a low-affinity binding site for OTF-1. On the other hand, if model A was correct, an Sp1 interaction should result in the same degree of transcriptional stimulation irrespective of whether it was combined with high- or low-affinity OTF-1-binding sites. Enhancer elements containing high- and low-affinity binding sites for OTF-1 either alone or in combination with Sp1-binding sites were, therefore, made. The wild-type enhancer element, located upstream of the *Sma* I site at position −198 (U2M:Sp1, Octa), was subsequently replaced by these mutated enhancer elements (Fig. 2). All of these constructions contained the same marked U2 maxigene (U2M) that was used for studies on U2 transcription (17). The first set of constructions contained a high-affinity binding site for OTF-1 (20), either alone (U2M:B) or with an Sp1-binding site (U2M:A). In the second set, the U2M:C and U2M:D constructions (Fig. 2), the high-affinity OTF-1-binding site in U2M:A and U2M:B, was substituted for by a low-affinity motif (20). U2M:E contains an Sp1-binding site but lacked a binding site for OTF-1. An additional base pair, not found in the wild-type human U2 enhancer was inserted between the OTF-1- and the Sp1-binding sites in these constructions. This insertion was necessary to avoid the appearance of an octamer-like motif (ATGCCCAA) on the opposite strand in the U2M:A and U2M:C constructions. The activity of the wild-type enhancer was unaffected by this alteration (unpublished observation). The transcriptional activities of the constructions were tested by transfections into HeLa cells followed by S1 analysis of the U2M transcripts (17). The U2M:A and the U2M:E constructions were first tested with the U2M:Sp1,Octa template and a construction lacking the U2 enhancer (U2M:−Enh, ref. 17). The transcriptional activity of the U2M:A construction was comparable to that of the U2M:Sp1,Octa template (Fig. 3*A*, lanes 4 and 5), whereas the transcriptional activity of the U2M:E construction indeed was very low (Fig. 3*A*, lane 6). However, a small amount of transcription was detectable from the U2M:E template after long exposure times (data not shown) whereas no transcription was seen when RNA extracted from either mock-transfected cells (Fig. 3*A*, lane 2) or from cells transfected with the U2M:−Enh construction (Fig. 3*A*, lane 3) was analyzed. There was a linear correlation between the amount of S1 nuclease-protected U2M probe detected and the amount of U2M:A DNA transfected in these experiments (data not shown). The transcriptional activities of constructions U2M:A–E (Fig. 3*B*) are summarized in Fig. 2. The

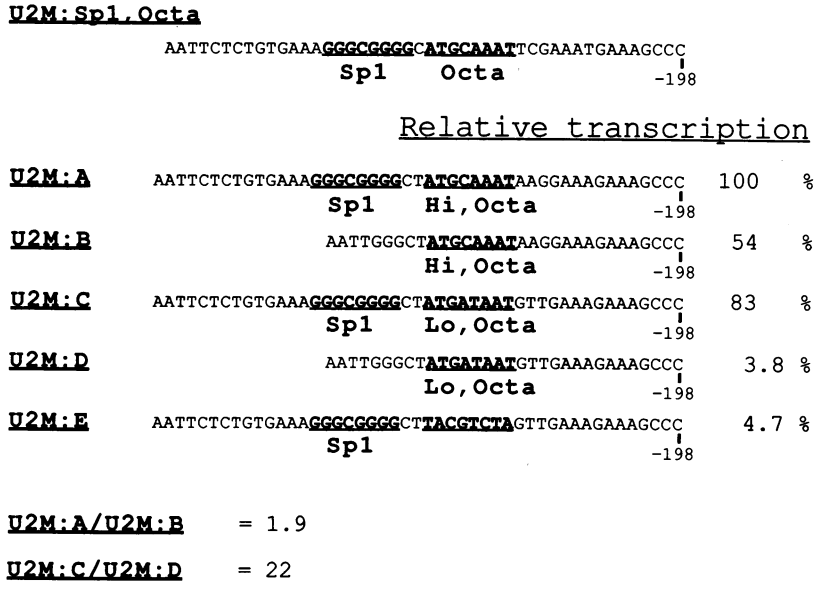

FIG. 2. Structure and transcriptional activities of enhancer constructions that contain high- or low-affinity binding sites for OTF-1. Sequences located upstream of position −198 of a marked human U2 snRNA maxigene are shown. U2M:Sp1,Octa contains the OTF-1- and the adjacent Sp1-binding site of a wild-type human U2 snRNA gene enhancer element. U2M:A–E comprise a set of constructions containing mutated enhancer elements consisting of an Sp1-binding site alone (U2M:E) or a high (U2M:A and -B)- or a low (U2M:C and -D)-affinity binding site for OTF-1 either alone (U2M:B and -D) or with an Sp1-binding site (U2M:A and -C). All U2M: constructions are identical apart from the sequence variations shown. Relative transcriptional activities are indicated.

transcriptional activity of the U2M:A construction was considered to be 100%. The U2M:B construction, which lacked the Sp1-binding site, showed a two-fold reduction in transcriptional activity. A dramatically different result was obtained when the transcriptional activities of the U2M:C and the U2M:D constructions were compared. A 20-fold stimulation of the U2 transcription was seen when an Sp1-binding site was included with a low-affinity OTF-1-binding site in the U2M:C construction. The template activity of construction U2M:D, which contained the low-affinity OTF-1-binding site alone, was only about 4% of that of U2M:A and comparable to that of construction U2M:E, which lacked an OTF-1-binding site but contained a binding site for Sp1.

The Functional Cooperation Between Sp1 and OTF-1 Is Dependent on the Distance Between Their Binding Sites. One prediction from model B in Fig. 1 is that the cooperative interactions between Sp1 and OTF-1 should be influenced by the distance between their binding sites. Constructions U2M:C5, U2M:C10, and U2M:C15 (Fig. 4) were made in which the low-affinity binding site for OTF-1 and an Sp1-binding site was combined at different distances from each other. A similar protocol has been used by Takahashi *et al.* (21) to study interactions between the simian virus 40 promoter and enhancer. The constructions were transfected into HeLa cells (Fig. 5) and the relative transcriptional activities were compared with those of the U2M:C and U2M:D constructions (Fig. 4). The insertion of 5 bp between the OTF-1- and Sp1-binding sites (template U2M:C5) resulted in a 2-fold reduction in the transcriptional activity (Figs. 4 and 5, lane 4). A 10-bp insertion between the binding sites (U2M:C10) resulted in a less-pronounced reduction in transcriptional activity (Figs. 4 and 5, lane 5) whereas the insertion of 15 bp between the sequence motives (U2M:C15) resulted in a 5-fold reduction in the transcriptional activity (Figs. 4 and 5, lane 6). The U2M:C15 construction was, however, more actively transcribed than the U2M:D construction, which lacks any Sp1-binding site.

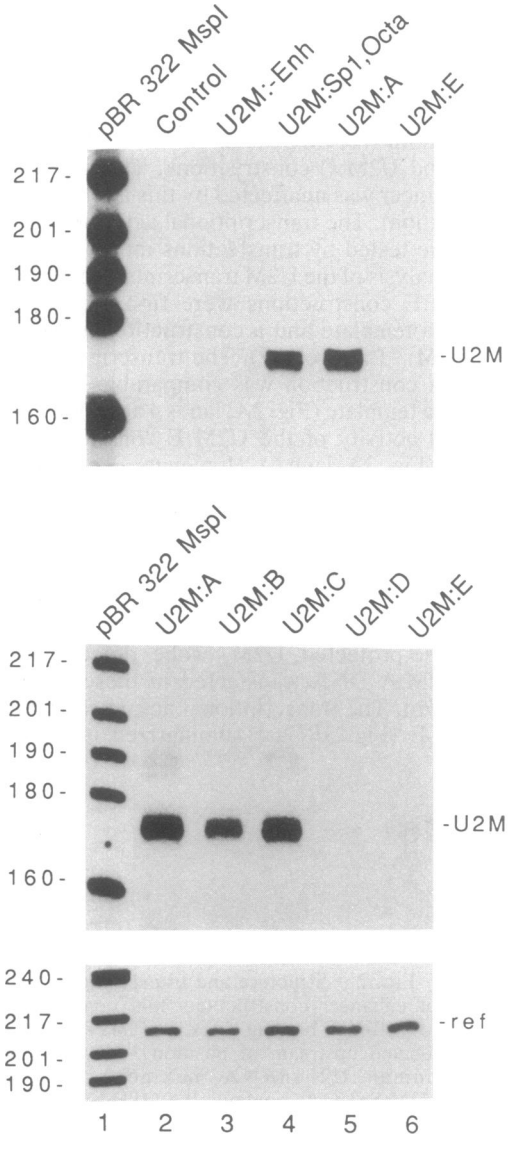

FIG. 3. Transcription of the U2M: high- or low-affinity OTF-1-binding site enhancer constructions in HeLa cells. Quantitative S1 nuclease analysis of transcripts from the various U2M: constructions shown in Fig. 2 and the reference plasmid pSXβ+ after transfections into HeLa cells. *Msp* I-digested pBR322 DNA was used as size markers. Fragments corresponding in length to correctly initiated U2M transcripts as well as β-globin transcripts are indicated. Control lane, cells transfected with the vector plasmid pM13+.

Gel Mobility Shift Experiments Reveal Cooperative DNA Interactions Between OTF-1 and Sp1 *in Vitro*. Gel mobility shift experiments were performed to test whether cooperative DNA interactions between OTF-1 and Sp1 could be detected *in vitro*. The U2M:A probe was incubated with nuclear extract from HeLa cells (19) either in the absence (Fig. 6*A*, lane 1) or in the presence (Fig. 6*A*, lanes 2–8) of various amounts of competitor DNAs. The amount of nuclear extract used in these experiments resulted in an almost complete, but still not saturated, occupancy of the U2M:A probe. Three major complexes were seen in this analysis, designated C1a, C1b, and C2 (Fig. 6). Approximately 90% of the probe was found in complex C2 when either no specific competitor (Fig. 6*A*, lane 1) or a 400-fold excess of NFI-specific competitor (Fig. 6*A*, lane 8) was added to the reaction mixtures. The C2 complex was specifically competed away by both Sp1- and OTF-1-specific competitors (Fig. 6*A*, lanes 2–7). In the presence of Sp1-specific competitor DNA, ≈75% of the probe was found in complex C1a (Fig. 6*A*, lanes 2 and 3) whereas, in the presence of OTF-1-specific competitor DNA, ≈60% of the probe was found in complex C1b (Fig. 6*A*, lanes 4 and 5). A minor complex that migrated slightly faster than the C2 complex could also be seen in some experiments (Fig. 6*A*, lanes 1 and 8). This complex was eliminated by both Sp1- and OTF-1-specific competitor DNAs and might thus result from interactions with partially degraded proteins. No complexes were seen when a mixture of Sp1- and OTF-1-specific competitor DNAs were added (Fig. 6*A*, lanes 6 and 7). When the U2M:A probe was incubated with increasing concentrations of nuclear extract, in the absence of any competitor DNA, complexes C1a and C1b appeared as a double-band at low extract concentrations. However, when the concentration of nuclear extract was increased, there was a drastic increase in the amount of probe found in complex C2 to the level where almost all of the probe is found in this complex (data not shown). We interpret the results in the following way: the C1a complex results from the binding of OTF-1 alone to the probe, whereas the C1b complex results from interactions between Sp1 and the probe. The C2 complex, lastly, consists of templates to which both Sp1 and OTF-1 have bound. The identity of the C2 complex was confirmed by methylation interference experiments (data not shown).

If OTF-1 and Sp1 interact with the U2M:A probe independently, one would expect to find ≈45% (75% × 60%) of the probe in the C2 complex instead of the actual 90%. This result thus implies that Sp1 and OTF-1 cooperate in their binding to the DNA template. The U2M:C oligodeoxyribonucleotide was used as a probe under identical experimental conditions (Fig. 6*B*). In the absence of any sequence-specific competitor DNA (Fig. 6*B*, lane 1), ≈35% of the probe was found in

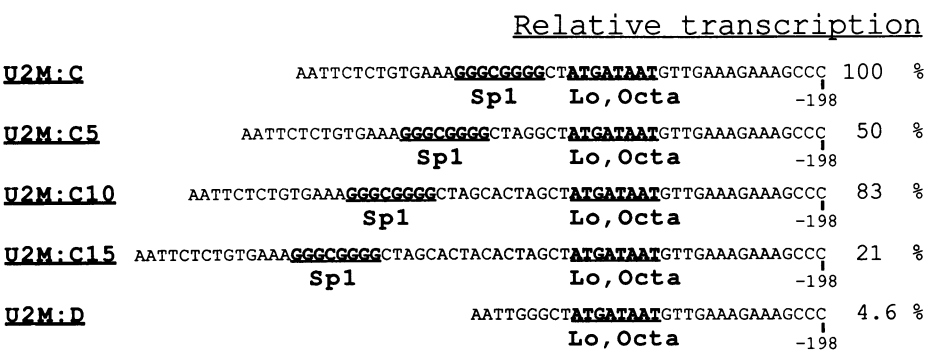

FIG. 4. Structure and transcriptional activities of enhancer constructions containing 5, 10, and 15 bp inserted between the low-affinity OTF-1- and the Sp1-binding site of the U2M:C construction. Relative transcriptional activities measured by S1 nuclease protection analysis of U2M transcripts are indicated.

complexes C1a and C1b, 35% was found as unbound probe (F), and 30% was found in complex C2. Although it was impossible to distinguish between the complexes C1a and C1b, when the densitometric scanning of lane 1 was performed, it appeared from a visual inspection that nearly all of the probe was found in the C1b complex. Only 3.5% of the probe was found in the C1a complex when a 400-fold excess of the Sp1-specific competitor DNA was added to the reaction mixture (Fig. 6*B*, lane 2), whereas ≈60% of the probe was found in complex C1b when the same amount of an OTF-1-specific competitor was added (Fig. 6*B*, lane 3). No specific complexes were seen when a mixture of these two competitor DNAs were added to the same reaction mixture (Fig. 6*B*, lane 4). The reduced amount of probe (3.5%) found in the C1a complex (Fig. 6*B*, lane 2) was expected since the U2M:C probe contains a low-affinity OTF-1 binding site. Based on the amount of C1a and C1b complex recovered after competition with the OTF-1- and Sp1-specific oligodeoxyribonucleotides, one would expect to find ≈2% of the probe (3.5% × 60%) in the C2 complex in the absence of any cooperative interactions. However, the results showed that ≈30% was recovered in the C2 complex. Gel mobility shift experiments were also performed using the U2M:D and U2M:E oligodeoxyribonucleotides as probes (data not shown). The relative amount of U2M:D probe found in an OTF-1-specific complex as well as the relative amount of U2M:E probe found in an Sp1-specific complex was the same as the amount of U2M:C probe found in the C1a and the C1b complexes in the presence of Sp1- or OTF-1-specific competitors.

The U2M:C15 probe was analyzed in a similar way (Fig. 6*C*). The results were very similar to those obtained with the U2M:C probe except for the amount of C2 complex recovered after incubation in the absence of any competitor DNA. With the U2M:C15 probe only 6% of the probe was found in the C2 complex (Fig. 6*C*, lane 1). This represents 20% of the amount of U2M:C probe found in the C2 complex under identical experimental conditions. The insertion of 15 bp between the OTF-1- and the Sp1-binding sites has apparently affected the interactions between OTF-1 and Sp1.

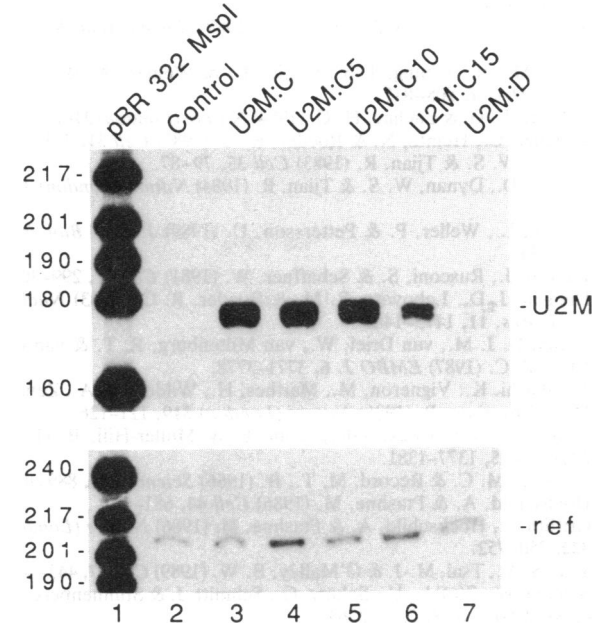

FIG. 5. Transcription of the enhancer constructions with variable distance between the Sp1- and OTF-1-binding sites in HeLa cells. S1 analysis was used to quantitate U2M transcripts after transfection of the U2M: constructions shown in Fig. 4.

DISCUSSION

In the present study we have utilized a set of synthetic enhancer constructions in combination with a U2 snRNA gene promoter to examine whether the functional synergism between transcription factors Sp1 and OTF-1 involves cooperativity in binding. Two models, which are depicted in Fig. 1, were considered. If model B, the cooperative DNA-binding model, was correct, the relative importance of the Sp1 interactions would be expected to differ in constructions where Sp1-binding sites were combined with high- or low-affinity binding sites for OTF-1. This result was obtained when a series of enhancer constructions were tested (Fig. 3). A 2-fold stimulation of transcription was seen when an Sp1-binding site was combined with a high-affinity OTF-1-binding site whereas a 20-fold stimulation was seen when it was combined with a low-affinity binding site for OTF-1. We have shown that transcription from a wild-type U2 RNA gene was reduced 5-fold when the Sp1-binding sites were deleted from the wild-type U2 enhancer (17). This result fits with our model since the OTF-1-binding site in the wild-type U2 enhancer can be presumed to have an affinity for OTF-1, intermediate to that of U2M:A and U2M:C, according to Pruijn *et al.* (20).

Our results demonstrate, furthermore, that the level of activation was dependent on the distance between the OTF-1- and the Sp1-binding sites. Thus these observations suggest that the functional cooperation between Sp1 and OTF-1 involves physical interactions between the two transcription factors. To demonstrate cooperative interactions *in vitro* between Sp1 and OTF-1 a series of gel mobility shift experiments were performed using the various enhancer constructions as probes. There was a cooperative formation of an OTF-1- and Sp1-binding-site-specific protein–DNA complex on the U2M:A probe (Fig. 6*A*). Fig. 6*B* shows that the binding of OTF-1 to a binding site with low affinity was increased 10-fold in the presence of Sp1 interactions. The most likely explanation for these results is that OTF-1 and Sp1 bind cooperatively. This interpretation was further strengthened by the observation that the level of OTF-1-binding decreased drastically when a 15-bp segment was inserted between the OTF-1- and Sp1-binding sites (Fig. 6*C*). The results do not

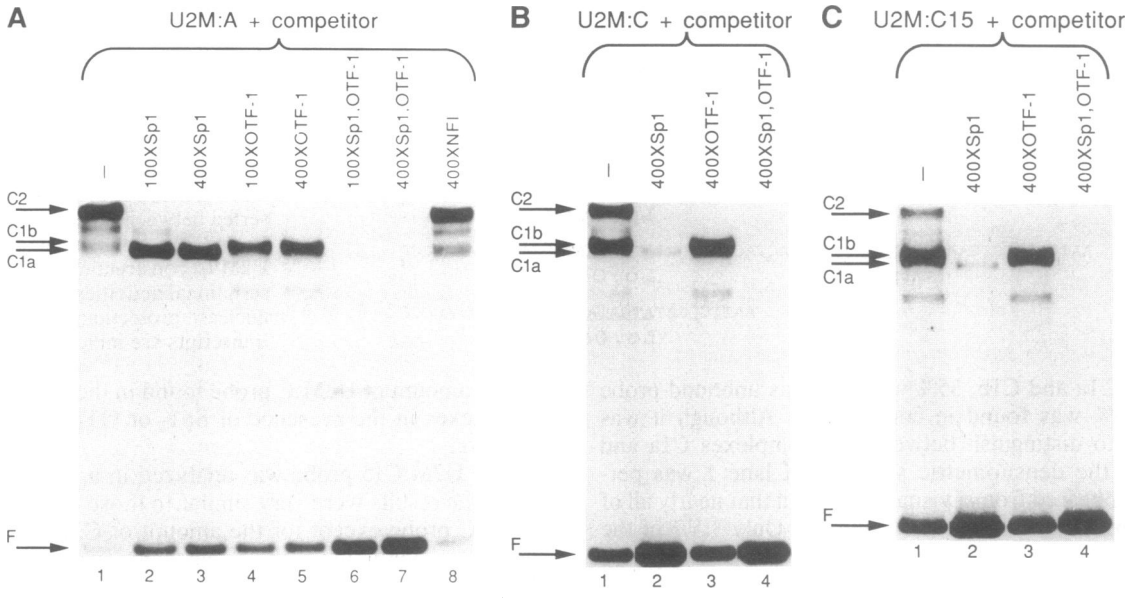

FIG. 6. Gel mobility shift analysis of oligonucleotides containing various OTF-1- and Sp1-binding sites. The oligodeoxyribonucleotides used for generating the enhancer constructions U2M:A (*A*), U2M:C (*B*), and U2M:C15 (*C*) (see Figs. 2 and 4) were used as probes. The molar excess of OTF-1-, Sp1-, or NFI-specific competitor oligodeoxyribonucleotides were as shown. The positions of the sequence-specific protein–DNA complexes (C1a, C1b, and C2) and free probe (F) are indicated.

completely rule out the possibility that Sp1 displaces another protein that interferes with the interaction between OTF-1 and its binding site. This alternative seems, however, improbable since such an interaction would result in a protein–DNA complex that should be detectable in the absence of Sp1 interactions. No such complex was observed (Fig. 6*A*). It should also be pointed out that the observed cooperativity in binding to the DNA template does not exclude that Sp1, in addition to facilitating the binding of OTF-1, makes additional contacts with the transcriptional machinery. Cooperative binding of regulatory proteins to DNA templates is a well-known phenomenon and the binding of regulatory proteins to adjacent sequence motives is an important feature of prokaryotic transcriptional regulation (22–25). These cooperative interactions allow for the expression of genes to be regulated by subtle changes in concentration of DNA binding proteins. In higher eukaryotes the role of cooperativity in transcriptional regulation is less well established although examples of cooperative interactions between mammalian transcription factors have been described. For instance, the interactions of glucocorticoid and progesterone receptors with adjacent hormone response elements are cooperative (26, 27), as well as the binding of the lymphoid-specific transcription factor OTF-2 to the octamer and the adjacent heptamer motives of an immunoglobulin heavy chain gene promoter (28, 29). The results presented in this communication demonstrate that Sp1 can influence the binding of another transcription factor, OTF-1, thus unraveling a mechanism by which Sp1 can modulate transcription.

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