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NonO enhances the association of many DNA-binding proteins to their targets

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ABSTRACT

NonO is an unusual nucleic acid binding protein not only in that it binds both DNA and RNA but that it does so via functionally separable domains. Here we document that NonO enhances the binding of some (E47, OTF-1 and OTF-2) but not all (PEA3) conventional sequence-specific transcription factors to their recognition sites in artificial substrates as well as in an immunoglobulin V_H promoter. We also show that NonO induces the binding of the Ku complex to DNA ends. Ku has no known DNA sequence specificity. These enhancement of binding effects are NonO concentration dependent. Using the E box activity of E47 as a model, kinetic studies demonstrate that the association rate of the protein–DNA complex increases in the presence of NonO while the dissociation rate remains the same, thereby increasing the sum total of the interaction. Oligo competition experiments indicate that NonO does not contact the target DNA in order to enhance the binding activity of DNA binding proteins. Rather, methylation interference analysis reveals that the induced E47 binding-activity has the same DNA-binding sequence specificity as the normal binding. This result suggests that one of the effects of NonO is to induce a true protein–DNA interaction. In this way, it might be possible for NonO to play a crucial role in gene regulation.

INTRODUCTION

NonO is a 55 kDa ubiquitously expressed protein originally identified as a non-POU domain-containing, octamer binding protein (1). Two nucleic acid binding domains are located in NonO: a tandem arrayed RNP (1,2) and a putative HTH followed by a region rich in both acidic and basic amino acid residues (1). NonO can bind via its HTH domain to double-stranded DNA (dsDNA) with restricted A/T specificity reminiscent of octamer motifs. It binds with less sequence specificity via its RNP domain to RNA (or single-stranded DNA). Binding of RNA and dsDNA can occur simultaneously indicating that the RNP and HTH domains function independently (1). NonO shares significant sequence identity with NonA, a Drosophila optomotor protein of unknown function (3,4), and with PSF, an essential mammalian splicing factor (5,6). The sequence homology among these three proteins is exclusively within the RNP domains and the adjacent charged region. NonO contains a stretch of glutamines near its N-terminus and a proline-rich region close to its C-terminus. Both types of domains have been shown to participate in protein–protein interactions required for the formation of transcriptional initiation complexes, and both are able to enhance transcriptional activity (7,8). Consistent with these features, we initially reported that NonO purified from a BCL_1 nuclear extract could transactivate an IgH promoter *in vitro* (9). However, recombinant NonO could not (Y.-S.Yang, unpublished results). We postulated that NonO might function in conjunction with other conventional DNA-binding proteins to stimulate the transcription (1).

Recently, three examples of DNA-binding enhancement by other proteins have been reported (10–12). In the absence of measurable protein–protein interaction, Phox1 (paired-like homeobox-containing protein) can induce DNA binding of SRF (serum response factor) to its target site by altering the association and dissociation rates of the SRF–DNA complex (10). The Tax protein, which activates transcription of the HTLV-1 LTR as well as other viral promoters, can stimulate the DNA binding activity of bZIP proteins. In this case the enhancement in binding is mediated by increasing essential bZIP dimerization (12,13). Finally, the high mobility group (HMG) protein I(Y) (14) can stimulate NF-κB or ATF-2 (activating transcription factor 2) binding to sites within the IFN-β promoter (11,15,16). It is proposed that contact of HMG I(Y) within the minor groove of the NF-κB binding site bends the DNA into a conformation more accessible for NF-κB/ATF binding within the major groove (11).

We report here that similar to the activity of these proteins, NonO increases the binding activities of OTF-1, OTF-2 and E47 to recognition sites, either engineered artificially on a duplex oligonucleotide, or present in their natural configuration in a promoter. This increased binding activity could be functional especially at a lower concentration of DNA-binding protein in that transcriptional activation can be induced by the binding of transcription factors to their target sites in the promoter or enhancer region. We hypothesize that through this kind of interaction NonO may be involved in the regulation of gene

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expression *in vivo*. The possible mechanisms for such enhancing activity will be discussed.

MATERIALS AND METHODS

Plasmid construction, protein expression and purification

The GST–E47(232C) fusion protein was constructed by the T4 DNA polymerase recession method (17). A PCR fragment, containing amino acid residue 232 to C-terminal of the E2A gene (18), was amplified by PFU DNA polymerase (Stratagene) and then subcloned into the *Bam*HI site of GST-VH, a modified pGEX-KG vector (19). This vector was engineered with a tobacco etch virus (TEV) protease cleavage site (20) at the 5′-end of the *Bam*HI site and a $(histidine)_6$ tag at the 3′-end of the *Bam*HI site. The expressed recombinant protein was either purified on Ni^{2+}–NTA beads (Quiagen) after removing its GST fusion portion by TEV protease cleavage and/or sequentially purified on Ni^{2+}–NTA beads after GST bead purification in order to obtain only the full-length products. Both GST–NonO and GST–E47 used in the studies were purified through GST and Ni^{2+}–NTA affinity columns. The recombinant NonO plasmid for yeast expression system was constructed by subcloning the PCR amplified, full length coding region of NonO DNA fragment into pHIL-D2 vector (Invitrogen); and then subsequently transforming *Pichia pastoris* for protein expression. The recombinant protein was engineered with a histidine tag at the C-terminus. The selection of NonO expressed yeast strains and protein purification protocols followed the manufacturer's instruction manual. The OTF-1 protein was produced by adding 1 µg OTF-1 plasmid DNA (pBSoct-1, a gift from Dr R.A.Sturm, Cold Spring Harbor) into an *in vitro* transcription/translation kit (Promega) using T7 RNA polymerase and the OTF-1 POU domain was a gift from Dr Wirth (Zentrum fur Molekulare Biologie Heidelberg, Germany). GST-PEA3 plasmid was a gift from Dr J.A.Hassell, McMaster University. Recombinant Ku proteins were expressed and purified from baculovirus as described previously (21).

DNA–protein binding assays

Conditions and probes used for mobility shift assays were as described (1). The *in vitro* translated OTF-1 product was diluted 8-fold and 1 µl protein was used in each binding reaction. For the enhancement studies, proteins were incubated together at room temperature for 10 min before the probe was added. The amount of protein used in each reaction is provided in the figures. For the on-rate and off-rate studies, scaled-up reactions were mixed together and 20 µl aliquots were removed and loaded onto the gel at time periods indicated on the figures. The gels were continuously running during the incubation time. After electrophoresis, the wet gels were dried and scanned by a Betagen (Betascope 603) or phosphoimager (Molecular Dynamics) and the complex intensity was summed and plotted. Conventional autoradiography was also performed on dried gels. For these studies, NonO cleaved from GST and purified as described above was utilized. For the PEA3 binding study, a double-stranded oligonucleotide probe containing the PEA3 binding sites (22), GATCCAGGAAGTGACCTAGGA-AGTAAGTAACG, was end-labeled with Klenow and [α-^{32}P]-dCTP. Other oligonucleotide sequences used are: PEA3-E47, GATCCAGGAAGTGACCTCAGGTGTAAG and reverse strand GGTCCTTCACTGGAGTCCACATTCATTGC; BN-PEA3, TAAGATGTACCCTGTCTCATGAATATGCAAATAGGAAG-AGTCTA and reverse strand TACATGGGCAGAGTACTTATA-CGTTTATCCTTCTCAGATACCA.

Antibody inhibition of protein–DNA interactions

For the antibody inhibition studies, 15 µg GST–NonO was preincubated with 15 µg anti-NonO polyclonal antibody at room temperature for 30 min. Antibody complexes were then precipitated with protein-A agarose beads, and a volume of supernatant equivalent to 1 µg GST–NonO fusion protein was added into a DNA–protein binding experiment described above.

Methylation interference analysis

A DNA fragment containing the BN sequence was end-labeled by Klenow and [α-^{32}P]dCTP. Single end-labeled DNA was subsequently modified by dimethyl sulfide at G residues and served as a probe in the following EMSA analysis. The protein–DNA complexes from eight tubes of reaction samples containing either 100 ng E47 or 25 ng E47 with 480 ng NonO were separated on 5% polyacrylamide gels. The bounded DNA probe as well as free probe were excised, electroeluted and ethanol precipitated. After cleavage with 10% piperidine, DNA samples were lyophilized and separated on 6% denaturing polyacrylamide gels.

RESULTS

NonO enhances the binding of several DNA-binding proteins

Previously we demonstrated that NonO exhibited specific DNA-binding activity for a fragment (BN) from the BCL_1 V_H promoter (1). While studying putative interactions between NonO and other promoter-binding proteins, we found that NonO increased the binding activity of both E47 and OTF-1 to their target sequences located within the BN fragment. The promoter fragment contains an E box site immediately adjacent to the 3′-end of the octamer sequence. The sequence CAGGTG is a perfect consensus binding site for class I bHLH-containing proteins such as E47. As shown in Figure 1A (lanes 1–6), formation of an E47 complex with the BN probe is E47 protein concentration-dependent. The HLH protein must dimerize in order to recognize its binding site (23). At low protein concentrations, the E47 homodimer cannot form measurable protein–DNA complexes (Fig. 1A, lanes 5 and 6). However, under the same conditions in the presence of GST–NonO, complex formation is greatly enhanced (Fig. 1A, lanes 10 and 11). These complexes are not altered in size as compared with those formed with higher concentrations of E47 alone. Rather, they show increased signal intensity.

To control for the non-specific activity, we replaced NonO with other proteins (GST or GST–E12) obtained by the same purification protocol. As shown in Figure 1B, we observed no consistent effect on the enhancement of DNA binding activity with other proteins. A small increase in signal intensity with GST and GST–E12 over GST–E47 alone (Fig. 1B, compare lanes 4 and 5 to lane 1) is the result of a non-specific increase in protein concentration. Such modified binding activity was also seen with the interaction of *in vitro* translated octamer binding proteins, OTF-1 and OTF-2, to octamer/heptamer binding sites within the same DNA fragment (Fig. 1C and data not shown). Nonetheless, the presence of NonO substantially enhances the binding activity and the induction is NonO-dependent.

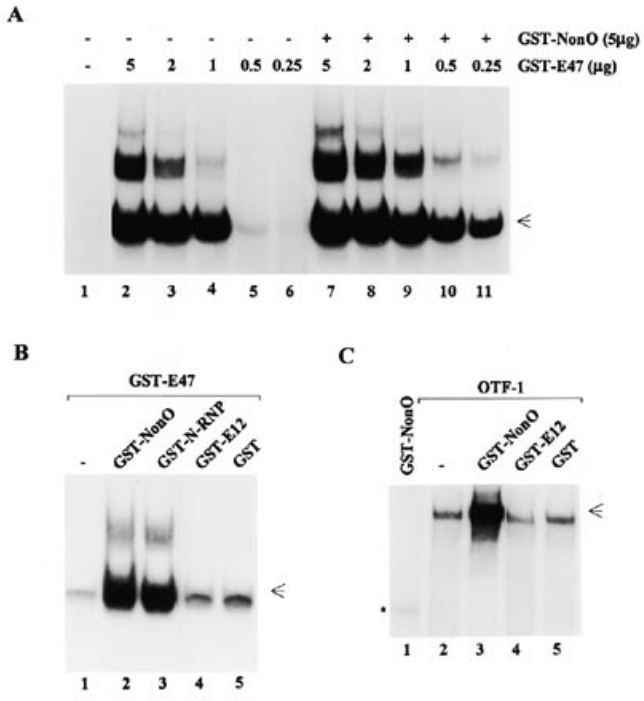

Figure 1. GST–NonO enhances the binding of OTF-1 and GST–E47 to their target sites in the BCL_1 V_H promoter. A DNA fragment containing the BN oligonucleotide sequence (1) was used as the probe. (**A**) GST–NonO enhances the binding of GST–E47 to its site. Lane 1 is probe alone. Lanes 2–6 are decreasing concentrations of GST–E47. Lanes 7–11 are the same as lanes 2–6 except that 5 μg GST–NonO has been added into the reaction mixture. The arrow denotes the monomeric E47–DNA complex. (**B**) Enhanced E47 binding depends on NonO. GST–E47 (0.5 μg) was mixed with 5 μg of either GST–NonO, GST–N-RNP, GST–E12 or GST protein and then tested by EMSA. (**C**) GST–NonO enhances OTF-1 binding at a low concentration of OTF-1. Lane 1 is GST–NonO alone binding to the V_H probe. * denotes the GST–NonO alone binding band. Lanes 2–5 are the same as in (**B**) except that 1/8 μl of *in vitro* translated OTF-1 was used as the protein source. The arrow marks the monomeric OTF-1–DNA complex.

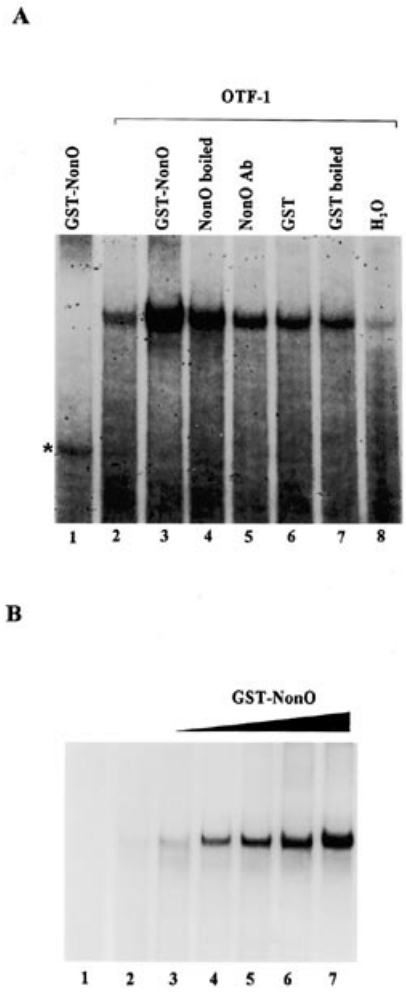

Figure 2. The enhancement of binding activity depends on NonO. (**A**) *In vitro* translated OTF-1 was incubated with the BN probe alone or was mixed with other proteins (as indicated in figure) prior to the binding reaction. Lane 1, 5μg GST–NonO alone (* denotes the NonO–DNA complex); lane 2, OTF-1 alone; lane 3, 1 μg GST–NonO plus OTF-1. Lane 4 is identical to lane 3 except that GST–NonO has been denatured by boiling. Lane 5 is the same as lane 3 except that the GST–NonO protein has been pre-absorbed by anti-NonO antibody. Lane 6 is 1 μg GST added to the reaction and in lane 7 is added 1 μg boiled GST. Lane 8 is H_2O as a control. (**B**) The increase in OTF-1 binding activity is NonO concentration dependent. Lane 1, probe alone; lane 2, OTF-1 alone; lanes 3–7 are OTF-1 incubated with various amount of GST–NonO prior to the start of the binding reaction. The GST–NonO concentration is 0.25, 0.5, 1, 2 and 5μg, respectively.

It is important that the observed stimulation of binding activity is actually dependent on NonO and the DNA-binding protein, not through the GST moiety fused to the interested proteins. Therefore, we produced OTF-1 in a reticulocyte lysate *in vitro* translation system as an alternative source for the DNA-binding protein and again tested the enhancing activity. The same result was obtained. As shown in Figure 2A, the OTF-1 binding activity is increased by the addition of NonO. However, adding either the fractions to which NonO has been pre-absorbed by rabbit polyclonal antibodies or denatured (by boiling) NonO, the enhancing activity is diminished (Fig. 2, lanes 4 and 5). We suspect that the modest increases in binding, as in the case above (Fig. 1B, lanes 4 and 5), arise from an increase in protein concentration unrelated to NonO activity. Substituting NonO with GST or denatured GST does not produce any change in OTF-1 binding to the BN probe (Fig. 2A, lanes 6 and 7). These results suggest that NonO-dependent enhancement is specific and dependent on the presence of NonO. When the DNA-binding protein (OTF-1 or GST–E47) was tested at a low concentration and its binding activity measured, we observed an increase in DNA-binding activity corresponding to the concentration of NonO (Fig. 2B and see results below).

NonO's enhancing activity does not require contact between NonO and DNA

NonO via its HTH domain can weakly bind the octamer site within the BN probe used in the EMSA (1). We next explored whether the binding of NonO to DNA is necessary for the enhancing activity. When the DNA-binding domain of NonO was eliminated by deletion of the C-terminal half of protein, the enhancement activity was retained (Fig. 1B, lane 3). Enhancement does not rely on NonO's single-stranded DNA-binding capacity which still resides in this domain (N-RNP), because enhancement was not impaired by the addition of excess single-stranded DNA competitor (data not shown). Thus, neither

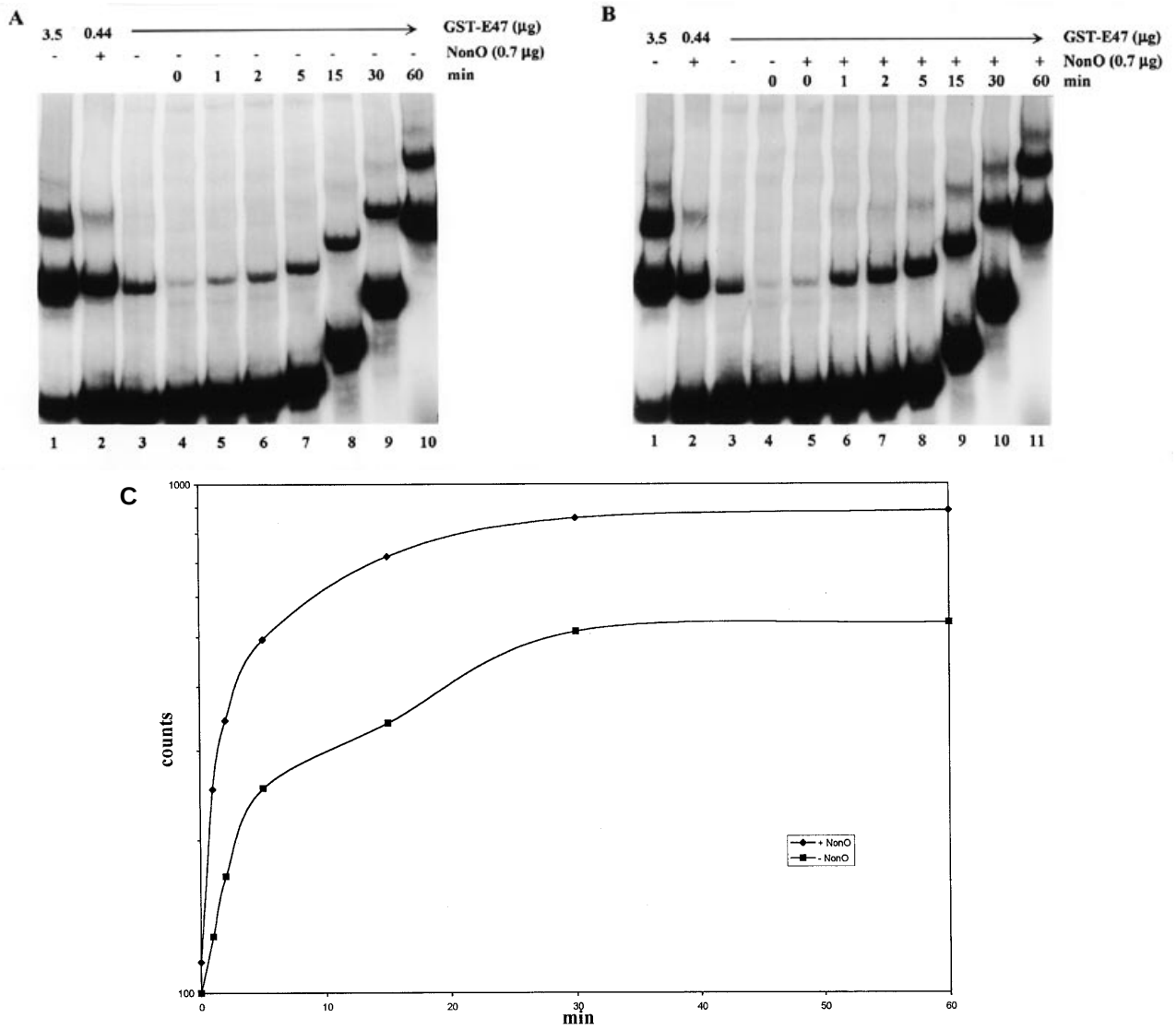

Figure 3. NonO accelerates the on-rate of E47–DNA complexes. (**A**) The on-rate of forming E47–DNA complexes without NonO. GST–E47 was mixed with the BN probe and aliquots were withdrawn at each time point and loaded onto a running EMSA gel. (**B**) The on-rate of forming E47–DNA complexes with NonO. GST–E47 was mixed with purified recombinant NonO at room temperature for 10 min before adding the BN probe. Aliquots were pipetted at the indicated times and loaded into an EMSA gel as in (A). (**C**) A plot of E47–DNA complex formation. The band intensities of E47–DNA complexes in the presence or absence of NonO were counted on a Betagen and were plotted versus the interaction time.

duplexed- nor single-stranded DNA binding of NonO to the DNA is necessary for its enhancing activity.

NonO enhances the association rate of the E47–DNA complex

In order to address the enhancement mechanism, we determined the effect of NonO on both the association and dissociation rates of E47–DNA complexes *in vitro*. First, we chose the lowest concentration of GST–E47 which showed NonO enhanced binding activity but still allowed measurable binding activity by itself (Fig. 3A, lanes 2 and 3). The ratio of NonO to GST–E47 used in this study was low (1–3-fold) in order to keep the intensity of the binding signal within a comparable range to GST–E47 alone. After mixing GST–E47 with the probe, aliquots were removed at various time points and analyzed (Fig. 3A, lanes 4–10). In a parallel experiment, identical conditions were achieved, except that recombinant NonO was added to the mixture 10 min before starting the reactions. As shown in Figure 3B, NonO enhanced E47 binding activity parallels the pattern seen with the E47 homodimer alone. In both situations the binding activity reaches a plateau after 30 min. However, in the presence of NonO within the first minute, E47 binding activity is nearly the same as after 5 min of incubation without NonO (compare Fig. 3B, lane 6 to Fig. 3A, lanes 5–7). When the intensities of the complex band are plotted versus the time of interaction, the faster on-rate of NonO plus GST–E47 than GST–E47 alone during the first 15 min of interaction is evident (Fig. 3C). Purified full length NonO without GST was employed in this experiment and shown to have the same enhancing characteristics as GST–NonO (Fig. 3A, lane 2 and Fig. 3B). These experiments exclude the possibility that the presence of GST in the fusion protein contributes to NonO's enhancing activity.

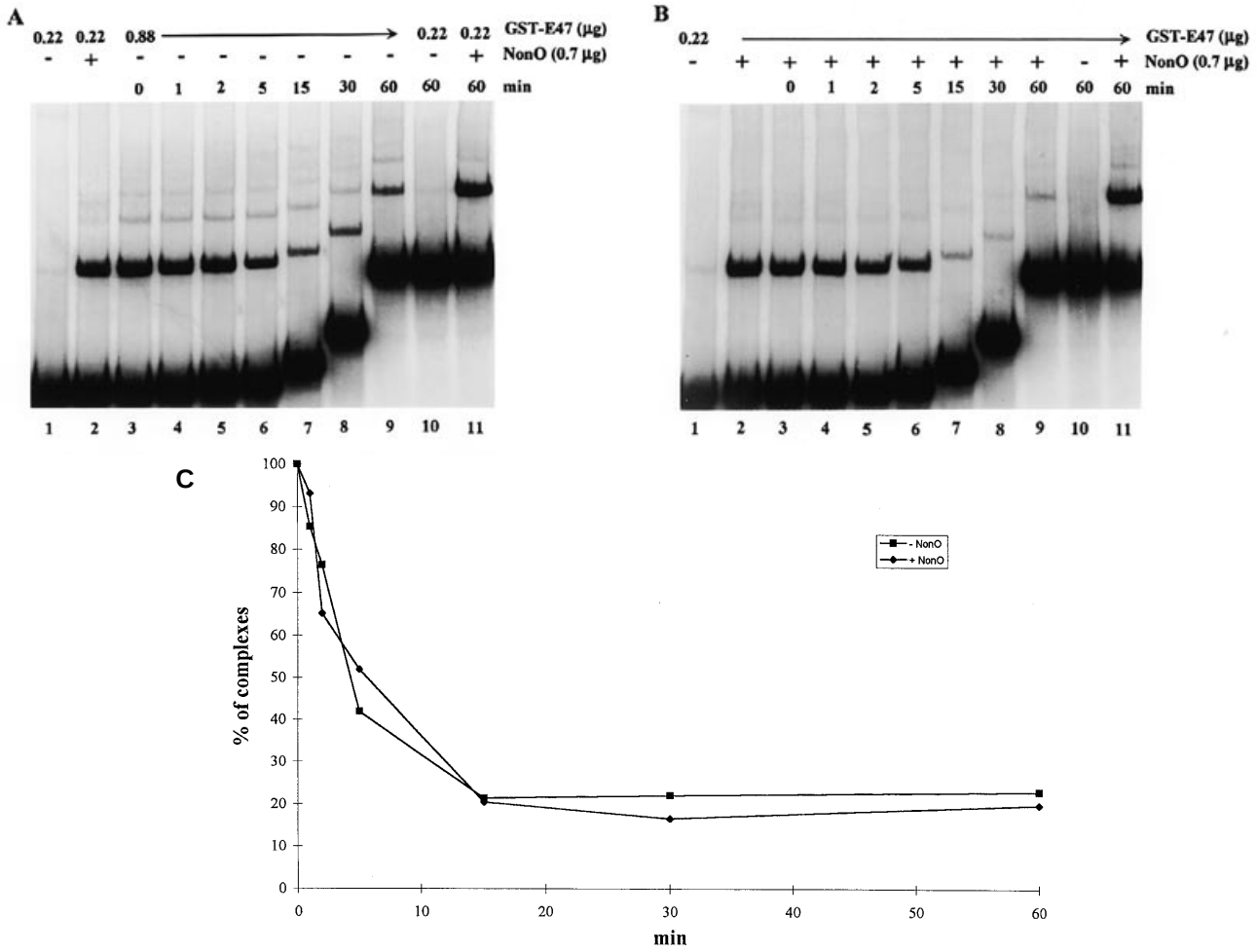

Figure 4. NonO has no effect on the dissociation rate of E47–DNA complexes. (**A**) The off-rate of the E47–DNA complex without NonO. GST–E47 was pre-bound to the BN probe for 30 min, then an excess of cold probe was added to the reaction to chase the labeled probe. Aliquots were withdrawn at time points indicated and loaded onto a running EMSA gel. (**B**) The off-rate of the E47–DNA complex in the presence of NonO. GST–E47, NonO and the BN probe were incubated together for 30 min before an excess amount of cold probe was added. Analysis was as in (A). (**C**) A plot of the E47–DNA complex dissociation rate. The protein–DNA radioactive EMSA complexes in (A) and (B) were scanned on a Betagen, and the counts were plotted as the percent complex remaining versus reaction time.

To study the dissociation rate, we chose the lowest concentration of GST–E47 which provided a significant enhancement of the binding activity by NonO (Fig. 4A and B, lanes 1 and 2). In the absence of NonO, 4-fold more GST–E47 is required to obtain the same binding intensity as compared with GST–E47 binding activity in the presence of NonO (compare lanes 2 and 3 in Fig. 4). After initial binding, 500-fold excess of unlabeled probe was added to the reaction, and aliquots were withdrawn at various time points and immediately loaded onto gels (Fig. 4A and B, lanes 3–9). The intensity of the binding complex from each aliquot was plotted as the percent of complexes remaining in the reaction (assuming the amount of complexes at time 0 is 100%) versus reaction time. As shown in Figure 4C, the curves (with or without NonO) are superimposable, suggesting that the dissociation rate is the same under both circumstances. Lanes 10 and 11 in Figure 4A and B demonstrate that E47–DNA complexes are stable throughout the entire testing period (at least 60 min). These results suggest that NonO alters the interaction of E47 to its binding site by increasing its association rate without altering the stability of the protein–DNA complex.

NonO's enhancing activity results in sequence-specific binding of E47 to its target site

In order to eliminate the possibility that either GST or other contaminants that co-purified with NonO might cause the change in E47's binding activity, we used several different methods to prepare E47 and NonO. E47 was purified by protease cleavage of the fusion protein and then applied to a second affinity column, Ni^{++}–NTA to ensure a full length product. An alternate preparation of NonO was obtained by expressing and purifying it from a yeast system (Fig. 5A). As shown in Figure 5B and C, using these highly purified proteins isolated by entirely different techniques, we consistently observed the enhancing activity by NonO at a low concentration of E47 (Fig. 5B, lanes 4, 5, 7 and 8, and Fig. 5C). In Figure 5C, when we substitute varying amounts of BSA for NonO in the reaction with minimal amounts of E47 (25 ng), it is apparent that BSA does not enhance E47 binding activity while NonO does (compare lane 3 with lane 7). In all of these experiments 2 µg BSA was included in the reaction mixture to further reduce the impact of protein concentration on the

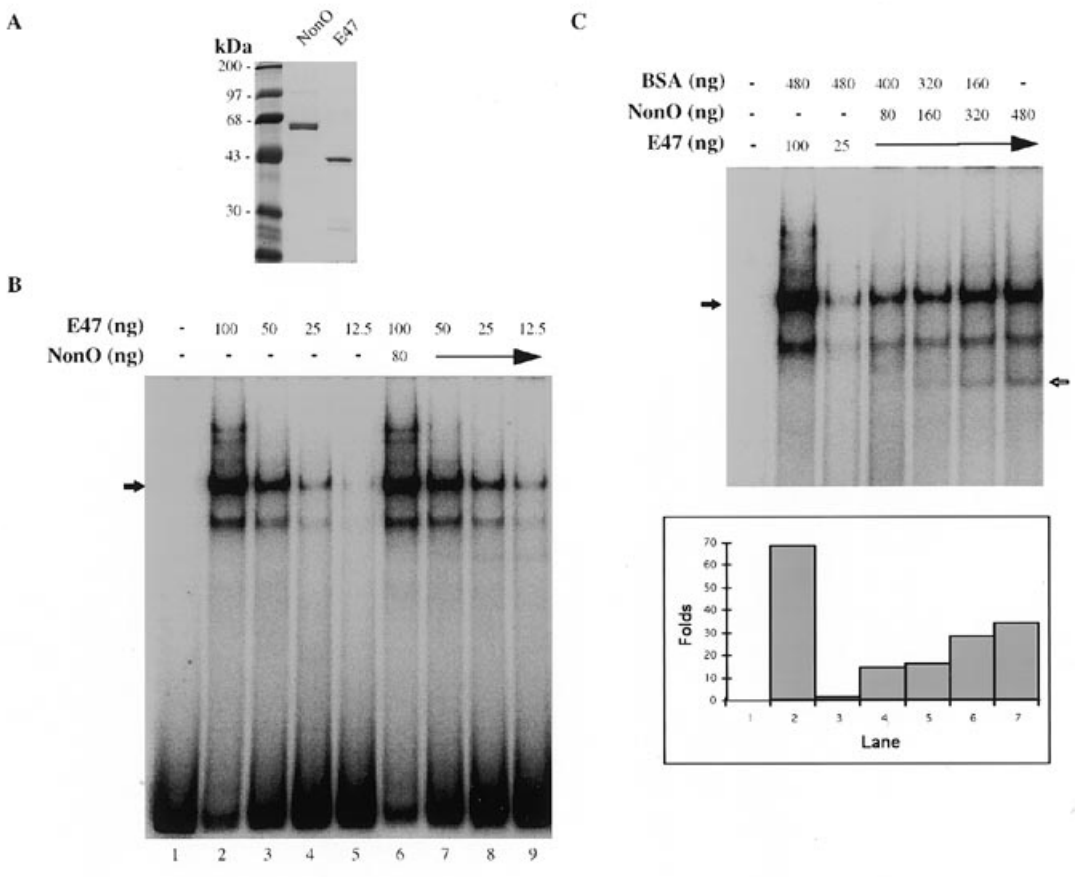

Figure 5. The enhancing activity is dependent on NonO concentration. (**A**) SDS–PAGE analysis of purified full length NonO and E47. Proteins (0.8 µg) were loaded onto SDS–PAGE gel and stained with Coomassie blue. (**B**) Recombinant NonO purified from a yeast expression system enhances E47 DNA-binding activity. Reaction mixtures were the same as in Figure 1A except that 2 µg BSA was included in each tube. Protein concentrations of E47 and NonO were as labeled. Arrowhead denotes the monomeric E47–DNA complex. The lower complex probably results from the binding of truncated E47 to probe. (**C**) The increase in E47 binding activity is NonO concentration dependent. Lane 1, probe alone; lane 2, E47 and BSA; lanes 3–7 represent E47 incubated with increasing amounts of NonO prior to the start of the binding reaction. BSA (2 µg) was included in all the reactions. Total protein concentration was further controlled by the addition of varying amounts of BSA into the reaction mixture tubes. The NonO and E47 concentrations are as indicated. The solid arrowhead indicates the E47–DNA complex and the open arrowhead denotes the NonO–DNA complex.

enhancing activity seen in Figure 1. With 25 ng E47 and NonO, the band signal was increased 35-fold over E47 alone, which we estimate to be the equivalent of two to three times as much E47 (Fig. 5C lower panel, lanes 2, 3 and 7). These results agree with our original observations that increased binding activity is dependent on the presence of NonO and is NonO concentration dependent. These experiments document that the source of NonO does not influence its enhancing activity.

To further address the specificity of induced E47 binding activity, we performed a methylation interference analysis of these induced E47–DNA complexes using the highly purified proteins. As shown in Figure 6, the complex from either normal (100 ng E47) or induced (25 ng E47 and 480 ng NonO) binding shows the same contacts of G residues in the E box binding sequence. This result argues that the enhanced binding activity seen with NonO does not alter its DNA-binding specificity. Since 25 ng E47 can barely form a measurable protein–DNA complex under the test conditions (Fig. 5C, lane 3) and with the addition of NonO the complex signal is increased, this result strongly suggests that genuine E47–DNA complexes are formed in the presence of NonO. NonO alone, however, does not generate the same interference pattern (data not shown).

NonO does not enhance the binding of all DNA-binding proteins

Having demonstrated the increased binding activity of E47 by NonO, we wondered whether NonO could likewise enhance the activity of PEA3, an Ets domain-containing transcription factor (22). The reason we chose this factor was because of the direct protein–protein interactions between NonO and PEA3 (unpublished data). As shown in Figure 7, the binding of PEA3 to its cognate site was not altered by NonO at a high ratio of NonO to PEA3, a circumstance under which an induction of binding activity with E47 was detected (Fig. 5C, lane 7). Therefore, NonO's enhancement of DNA-binding activity is not a general phenomenon.

The enhancing activity of NonO does not require direct contact between NonO and DNA

Since the probe used in the original studies contained a heptamer, an octamer and an E box binding site [BN-($H^+O^+E^+$)], one possibility for the enhancing activity was that NonO directly bound to the probe (1) and subsequently altered the structure of the DNA for the DNA-binding proteins to bind as has been reported for HMG I(Y) (11,15,16). To explore this possibility we

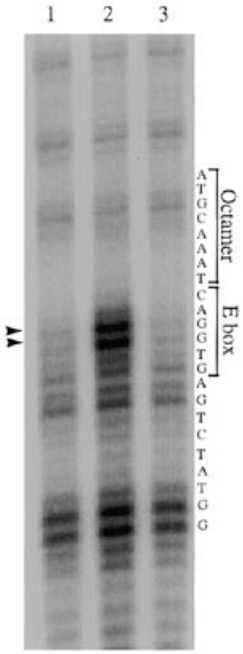

Figure 6. Methylation interference analysis of the E47 binding site. Lane 1, 25 ng E47 plus 480 ng NonO in the binding reaction; lane 2, free probe; lane 3, 100 ng E47 alone in the binding reaction. Octamer and E box sites are as indicated. Methylation interfered G residues inside E boxes are marked. The other strand of methylation interference study was also done but revealed no differences among them.

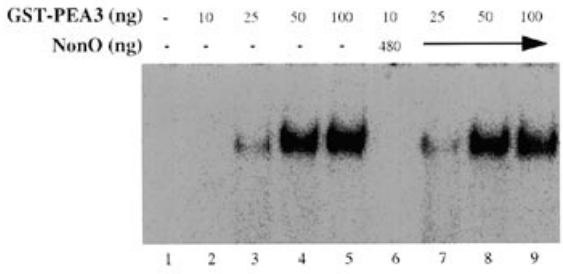

Figure 7. NonO does not enhance the DNA-binding activity of PEA3. Lane 1 is probe alone. Lanes 2–5 contain increasing amounts of GST–PEA3. Lanes 6–9 contain 480 ng NonO mixed 10 min before the addition of probe with varying amounts of PEA3 as indicated. BSA was added to reactions to control the total protein concentration as described in the legend of Figure 5C.

first tested the same probes but by altering their heptamer and octamer sites. As shown in Figure 8, the POU domain of OTF-1 binds to probes without a heptamer, BH ($H^-O^+E^+$) or octamer, BO ($H^+O^-E^+$) (1) and its binding activity still can be induced by NonO (Fig. 8, lanes 4–6 and 11–13). Both probes were also used for E47 binding studies and the same enhancing activity as the BN probe was seen (data not shown). These results suggest that neither heptamer nor octamer is required for NonO's enhancing activity.

However, the possibility still remains that NonO needed to contact its target site to display its activity. To address this we performed an oligo competition experiment. As shown in Figure 9A, while using the BH ($H^-O^+E^+$) probe for OTF-1 binding,

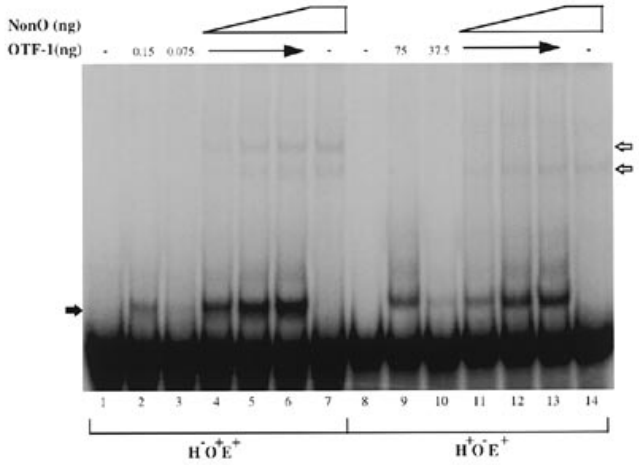

Figure 8. The POU domain DNA-binding activity to octamer or heptamer sites is enhanced by NonO. The BH ($H^-O^+E^+$) or BO ($H^+O^-E^+$) oligos were end labeled and used as a probe. Lanes 1 and 8 are probe alone. The amount of OTF-1 protein used in reactions is indicated at the top. 160, 320, 480 and 480 ng NonO were added to the reactions in lanes 4–7 and lanes 11–14 individually. The solid arrowhead denotes the complexes of POU domain and probe; open arrowheads indicate the complexes of NonO and probes. Total protein concentration was adjusted with BSA as described previously.

the excess amount of competitor containing E box and PEA3 binding sites, PEA3–E47 (P^+E^+) did not compete away the enhanced OTF-1 binding activity (lane 5). The reason we used this oligo as a competitor was our observation concerning the enhancing activity of NonO on E47 binding when it was used as the probe (Fig. 9B, lane 4). If binding site contact were necessary, we would expect that excess PEA3–E47 oligo should compete and abolish the enhanced binding activity of OTF-1. In a reverse experiment, the induced E47 binding activity with PEA3–E47 (P^+E^+) probe did not disappear when excess of octamer binding sites were present as the competitor (BN-PEA 3, $H^+O^+P^+$) (Fig. 9B, lane 5). In contrast, the binding of PEA3 to this probe did not show enhanced activity under similar conditions for E47 binding. In lane 6, in the presence of 480 ng NonO, there is no evidence of direct NonO binding. Therefore, these results suggest that contact between NonO and DNA is not necessary for its enhancing activity.

DISCUSSION

We demonstrated previously that NonO was a DNA-binding protein that displayed low affinity for the octamer motifs in the BCL_1 and V1 V_H promoters (1). Here we show that in addition to its own nucleic acid binding activity, NonO can alter the binding activity of other DNA-binding proteins by enhancing their association to their cognate sites. This enhancing activity could alter their subsequent function.

Phox1 and Tax are examples of proteins that have been shown previously to enhance DNA-binding activity (10,12). Phox1 enhances the interaction between SRF and its target site, SRE (serum response element), and Tax stimulates the binding of ATF (activating transcription factor) to its responsive site. Although Phox1 alone can bind to the SRE (similar to NonO's binding to the V_H promoter), its binding affinity is low (10). Tax, on the other hand, does not directly interact with DNA (12). Interestingly, both Phox1 and Tax are able to augment DNA-binding activity at low

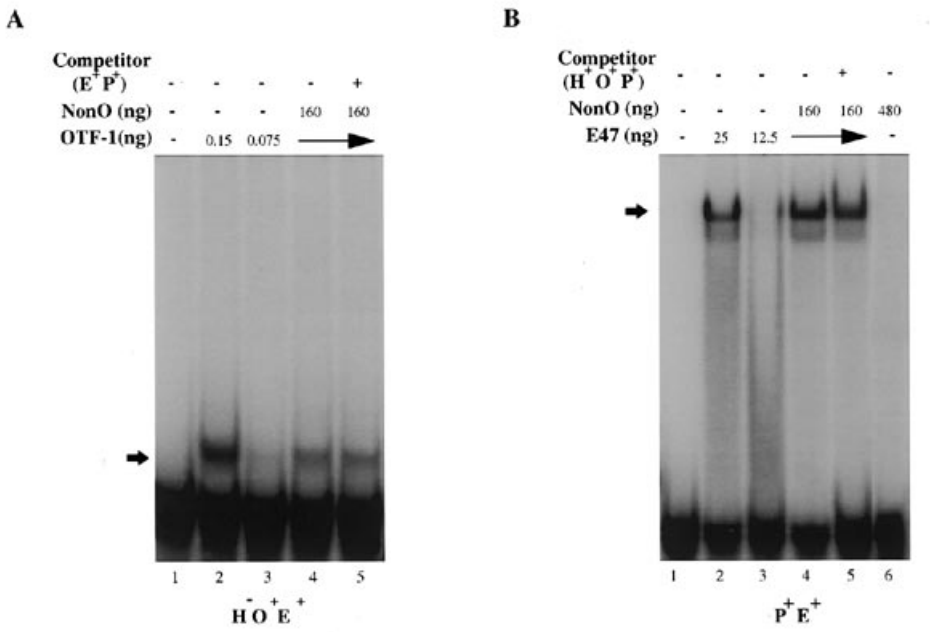

Figure 9. The enhanced binding activity of NonO does not require contact of NonO and DNA. (**A**) The enhanced POU domain binding activity is not competed by the E box containing oligo. The least amount of NonO needed in the reaction to observe the enhanced activity was used in the reactions. Lane 5, 250 ng PEA3–E47 (P^+E^+) oligo (250-fold excess) was added as a competitor before adding the probe. The amount of protein in each lane is as labeled. The solid arrowhead denotes the protein–DNA complexes. (**B**) The enhanced E47 binding activity is not competed by heptamer or octamer sites. PEA3–E47 was end-labeled as the probe. Lanes 1–4 are as labeled. Lane 5, 250 ng BN–PEA3 oligo (250-fold excess) containing functional heptamer and octamer sites was used as a competitor for the enhanced E47 binding activity. The amount of protein used in each lane is as labeled. The solid arrowhead denotes the E47–DNA complexes. Total protein concentration was adjusted by BSA as described in previous legends.

SRF or ATF concentrations. Our studies show that NonO's enhancement is most dramatic at low concentrations of E47 and the octamer binding protein, OTF-1. However, the binding of PEA3, an Ets-domain containing transcription factor (22), was not stimulated by NonO. Thus, the enhancement effect of NonO is not a general phenomenon.

We ascribe these characteristics to NonO alone, as when NonO was absorbed from the reaction with antiserum, or when NonO was heat-denatured prior to testing, no enhancing activity was seen. Various NonO sources either separated from the GST moiety or purified from a eukaryotic expression system gave the same enhancing activity as the GST–NonO fusion protein we reported previously. GST alone or other irrelevant proteins including BSA do not display an enhancing activity. All of these results suggest that one of NonO's intrinsic properties is its ability to enhance DNA-binding activity of many DNA-binding proteins.

The effect of NonO on E47 binding in some respects resembles the induction of ATF DNA-binding activity by Tax (12). ATF is a basic leucine zipper-containing (bZIP) protein that requires dimerization for binding. Tax stimulates this protein–protein interaction and reduces the free energy of formation of bZIP–DNA complexes (24), which in turn increases the association rate of the bZIP–DNA complex but not its dissociation rate. In our studies, the association rate of E47–DNA complexes is also enhanced by NonO. Unlike Phox1's influence on both the on- and off-rate of SRF–SRE complexes, the dissociation rate of E47–DNA complexes is not altered by NonO. This finding would support a Tax-like mechanism in which NonO induces the dimerization of E47 *in vitro* and subsequently stimulates its DNA-binding activity at a low concentrations. However, this interpretation is difficult to reconcile with the fact that DNA-binding of OTF-1, a protein that does not require dimerization for its biologic activity, is also enhanced by NonO under comparable conditions.

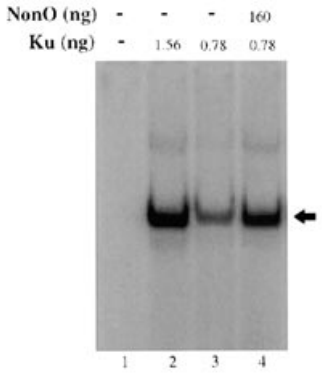

Figure 10. NonO increases the DNA end-binding activity of the Ku complex. The PEA3–E47 oligo was used as the probe in this experiment. At low concentration of Ku complex (0.78 ng), its end-binding activity was increased by the presence of 160 ng NonO (lane 4). The solid arrowhead denotes the Ku–DNA complex. Total protein concentrations were adjusted with BSA as described.

A second possible mechanism for enhancement is suggested by NonO's DNA binding affinity. NonO may interact with the DNA molecule first, and then alter local DNA structure, making it more accessible to specific binding proteins. This mechanism has been proposed for the induction of NF-κB or ATF-2 binding by HMG I(Y) (11). Binding of HMG I(Y) to the minor groove of the IRS site within the IFN-β promoter results in bending of the DNA,

presumably leading to better interaction. However, a truncated form of NonO which cannot bind double-stranded DNA can still stimulate E47–DNA binding. Although NonO interacts weakly with double-stranded DNA, its affinity for RNA or single-stranded DNA is much stronger. Such activity could bear on the enhancement effect reported here. Numerous single-stranded DNA or RNA binding proteins have been implicated in replication and transcription (25–29). However, NonO's enhancing activity is still apparent in the presence of single-stranded DNA competitors (data not shown). Furthermore, in competition analyses, the induced E47 binding activity still occurs in the presence of excess NonO binding sites (BN–PEA3). These results support the view that NonO's contact to its target DNA at their cognate sites is not required for its enhancing activity.

A third and favored mechanism is suggested by the primary structure of NonO. NonO has stretches of glutamines within the N-terminal region and a proline-rich region in its C-terminus (1). These types of domain have been shown to be involved in protein–protein interactions (7,8). Through interaction of these putative domains with DNA-binding proteins, NonO might increase their local concentration to a level that stimulates the formation of protein–DNA complexes. This would explain why the enhancing activity is maximal at lower DNA-binding protein concentrations. A third domain that can fulfill this function is suggested by the behavior of N-RNP. This enhancing-competent truncation mutant retains the tandem RNP domain (17). It has been documented that RNP domains are involved in protein–protein interactions (30). We have biochemical and genetic evidence that the dimerization involved in NonO is mediated, at least partially, by its RNP domains (unpublished data). As with full length NonO, N-RNP could recruit DNA-binding proteins and increase their local concentration, thereby stimulating protein–DNA interactions.

E47 and OTF-1 are well characterized transcription factors that can function either in a tissue-restricted or ubiquitous context (23,31–36). Their DNA binding sites or protein binding domains share no similarity (23,37,38); therefore, it is unlikely that there is a consensus sequence for their enhanced binding activity. This leads us to hypothesize that NonO exerts its effect by changing local protein concentration. This hypothesis is supported by our observation that NonO increases the DNA end-binding activity of Ku (Fig. 10, lane 3). The Ku complex is a well characterized DNA end-binding protein. Under similar circumstances at low concentration of Ku, NonO increases the amount of protein–DNA complexes *in vitro*. Most importantly, in this case, no sequence-specific DNA-binding activity is involved. Thus, we suggest that independent of the interaction between NonO and nucleic acids, NonO recruits DNA-binding proteins and provokes them to form a functional complex.

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