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Substitution of basic amino acids in the basic region stabilizes DNA binding by E12 homodimers

Stephen J. Vitola, Aifei Wang and Xiao-Hong Sun*

Department of Cell Biology, New York University Medical Center, New York, NY 10016, USA

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ABSTRACT

The E2A gene encodes two alternatively spliced products, E12 and E47. The two proteins differ in their basic helix–loop–helix motifs (bHLH), responsible for DNA binding and dimerization. Although both E12 and E47 can bind to DNA as heterodimers with tissue-specific bHLH proteins, E12 binds to DNA poorly as homodimers. An inhibitory domain in E12 has previously been found to prevent E12 homodimers from binding to DNA. By measuring the dissociation rates using filter binding and electrophoretic mobility shift assays, we have shown here that the inhibitory domain interferes with DNA binding by destabilizing the DNA–protein complexes. Furthermore, we have demonstrated that substitution of basic amino acids (not other amino acids) in the DNA-binding domain of E12 can increase the intrinsic DNA-binding activity of E12 and stabilize the binding complexes, thus alleviating the repression from the inhibitory domain. This ability of basic amino acids to stabilize DNA-binding complexes may be of biological significance in the case of myogenic bHLH proteins, which all possess two more basic amino acids in their DNA binding domain than E12. To function as heterodimers with E12, the myogenic bHLH proteins may need stronger DNA binding domains.

INTRODUCTION

The mammalian E2A gene encodes E12 and E47 transcription factors that contain a characteristic basic and helix–loop–helix motif (bHLH) (1), which is responsible for DNA binding and dimerization (2,3). As a result of alternative splicing of the E2A transcripts, E12 and E47 mRNAs differ in the exon that encodes the bHLH motif (4). Consequently, E12 and E47 proteins exhibit different DNA-binding properties: E47 homodimers bind to DNA very efficiently while E12 homodimers do so poorly. Both E12 and E47 can form heterodimers with other tissue-specific bHLH proteins such as the myogenic factors and MASH proteins, and these heterodimers play important roles in the differentiation of many cell types including muscle and neuronal cells (5–11). It has also become evident that the E2A gene products bind to DNA as homodimers in B lymphoid cells and control early B cell development (12–15). Therefore, understanding the function of E2A homodimers is of biological importance.

In examining the functional difference between E12 and E47, Sun and Baltimore have previously identified an inhibitory domain in a region N-terminal to the basic DNA binding domain of E12, which is not present in E47 (4). This inhibitory domain was shown to prevent E12 homodimers from binding to DNA efficiently. However, the mechanism by which the inhibitory domain prevents DNA binding is not known. The work presented here is intended to address the questions whether the inhibitory domain prevents the formation of DNA binding complexes or causes a fast dissociation of the binding complexes, and whether any factors exist that can alter the stability of the DNA binding complexes of E12 homodimers.

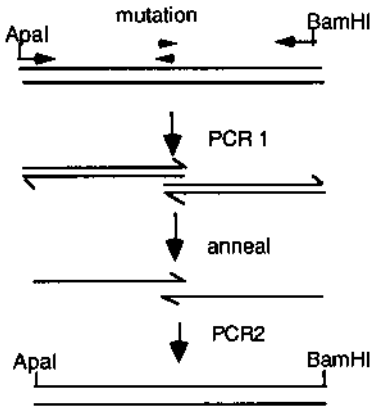
MATERIALS AND METHODS

Generation of mutants

To produce N-terminally truncated E12, a *Nde*I–*Bam*HI fragment from pE12N (4) was cloned into the *Nde*I and *Bam*HI sites of the PRSET-A vector (Invitrogen). This plasmid was used in all subsequent mutagenesis experiments. pE12-MyoDBJ was created by replacing an *Apa*I–*Bam*HI fragment in pE12N with a fragment that is produced through polymerase chain reaction (PCR) using oligonucleotides E12-Apa and E12-3′Bam as 5′ and 3′ primers and the E12-MyoD(BJ) plasmid as a template (a gift from Dr H. Weintraub; 16). Mutants 1–5 were generated through a two-step polymerase chain reaction (PCR) as diagrammed below in Scheme 1. Oligonucleotides E12Apa and E12-3′Bam were used as 5′ and 3′ primers in all of the above cloning experiments. For each mutation, a pair of complementary oligonucleotides was synthesized and designated as ‘F’ (forward) and ‘R’ (reverse) following the name of the mutation (their forward sequences are listed below). These primers were used in conjunction with E12-Apa and E12-3′Bam primers to create two mutant fragments through PCR using pE12N as a template. The two fragments were then denatured and annealed to be used as a template for a second PCR with E12Apa and E12-3′Bam as primers. The final PCR products containing specific mutations were cloned into the *Apa*I and *Bam*HI sites of pE12N.

To introduce various mutations at position 4 in the basic region of E12, PCRs were carried out using pE12N as a template, E12Apa as the 5′ primer and primer 2D, 2E, 2R or 2X as the 3′ primer. The PCR products were cloned into the *Apa*I and *Sma*I

* To whom correspondence should be addressed



Scheme 1.

sites of pE12N. Random mutations generated by primer 2X, which contains three random bases, were identified by sequencing. To inactivate the inhibitory domain in these various 2X mutants, primers E12mI (4) and E12-3′Bam were used in PCR with each 2X mutant as a template and the PCR product was cloned into the *Apa*I and *Bam*HI sites of pE12N.

Primers:

E12Apa	5′-CCCCGGGCCCGGACCAGC-3′
E12-3′Bam	5′-GATGCGGATCCTCACATGTGCCCGGCGGGGT-3′
M1F	5′-CAGAAGGCCACCACCAACGCTGATCGCCGGGTG-3′
M2F	5′-GAGCGCCGGAAGGCCAATAAC-3′
M3F	5′-CGGGAGCGGCGCCGGGTCCGT-3′
M4F	5′-CGCCGGGTGAAGAATAACGCC-3′
M5F	5′-GCCAATAACAAGCGGGAGCGG-3′
2X	5′-CTCCCGGGCGTTATTGGCNNNCCGGCG-3′
2D	5′-CTCCCGGGCGTTATTGGCGTCCCGGCG-3′
2R	5′-CTCCCGGGCGTTATTGGCCCGCCGGCG-3′
2E	5′-CTCCCGGGCGTTATTGGCCTCCCGGCG-3′
E12mI	5′-CCCCGGGCCCGGACCAGCCCAAACCAGAACCAGG-ACGAC-3′

Overexpression of proteins in *E.coli*

Expression plasmids were transformed into the *E.coli* strain BL21(DE3) which contains the T7 RNA polymerase gene under the control of the lacUV5 promoter (17). An overnight culture of freshly transformed bacteria was diluted 10-fold to a final volume of 5 ml. The culture was grown for 30 min and then induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.4 mM and incubation for an additional 3 h. Cells were then harvested and resuspended in a lysis buffer [10 mM Tris–HCl pH 7.5, 25 mM EDTA, 1% Triton X100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT)]. The suspension was frozen and thawed twice and NaCl was added to a concentration of 0.5 M (4). The suspension was sonicated and spun at 16 000 *g* for 10 min. The supernatant obtained at this step was used for binding reactions in the filter binding assays. Extracts were analyzed through electrophoresis on 13% SDS–PAGE and stained with Coomassie blue. Approximations of protein concentration were made from these gels and all expressed proteins were adjusted to a similar concentration.

In vitro transcription and translation

To produce the proteins using rabbit reticulocyte lysate, the DNA templates for *in vitro* transcription were generated by PCR using the T7 sequencing primer (Promega, Madison, WI) and the E12-3′Bam primer with each of wild-type or mutant expression plasmid as a template. The PCR products were phenol–chloroform extracted and ethanol precipitated. *In vitro* transcription reactions with T7 polymerase were set up using an aliquot of these products. RNAs were purified by phenol–chloroform extraction and ethanol precipitation. One fifth of the RNAs were used for *in vitro* translation using rabbit reticulocyte lysates and [^{35}S]methionine according to the vendor (Promega, Madison, WI). One microliter of each of the *in vitro* translation reactions was analyzed by electrophoresis using 13% SDS–PAGE, and the amount of product was quantified using PhosphoImager. The amount of proteins to be used for DNA binding studies was adjusted relative to that of E12N with appropriately diluted unprogrammed reticulocyte lysates.

Full-length E12 and E47 cDNAs were cloned into pGEM2 and pBluescript KS vectors respectively. The plasmids were linearized at the 3′-end of the cDNAs and used for *in vitro* transcription with T7 polymerase. RNAs were then used for *in vitro* translation as described above. Equal amounts of the E12 and E47 proteins were used for binding reactions.

Electrophoretic mobility shift assays (EMSA)

The κE2 site (TCGAGGCCACCTGCCTG) was cloned into the *Sal*I site of pUC18 (1) and the probe was prepared by labelling the ends of a 64 bp *Hin*dIII–*Eco*RI fragment with [α-^{32}P]dATP and [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. All binding reactions were performed using 1 µg poly(dI·dC), 10 000 c.p.m. of the probe, and equal amount of *in vitro* translated wild-type or mutant proteins in a binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT and 5% glycerol] with a total volume of 20 µl. Reactions were incubated at room temperature for 15 min. Binding reactions were analyzed by electrophoresis on 4% non-denaturing polyacrylamide gels in 0.5× TBE electrophoresis buffer (18).

To measure the off-rate of the binding complexes using EMSA, binding reactions were set up in a total volume of 200 µl with ten times of the amounts of *in vitro* translated proteins, and incubated for 15 min. After 20 µl of the binding reaction was loaded on the gel, 5 µg of the κE2 oligonucleotides was immediately added to the remainder of the binding reaction. Twenty µl aliquots were loaded onto the gel at a given time point while electrophoresis continued.

Filter binding assays to measure dissociation rates of DNA–protein complexes

To measure the dissociation rates, the binding reactions were performed in a total volume of 400 µl binding buffer containing ~2 µg E12N or E47N produced in *E.coli*, 10 µg poly(dI·dC) and 200 000 c.p.m. of the κE2 probe mixed in the order listed. The reactions were incubated at room temperature for 15 min to allow for binding to occur. A 20 µl aliquot for E47N or 40 µl aliquot for E12N was then passed through a nitrocellulose filter (type HA with a pore size of 0.45 µm, Millipore) followed by a 10 ml wash with ice cold binding buffer. This time point was designated t = 0. Five µg of the κE2 oligonucleotide competitor was then added.

After the incubation period of 1 min, 20 µl aliquots (or 40 µl for E12N) were withdrawn and assayed at 1 min intervals. The filters were air dried and counted in a scintillation counter to determine the amount of protein–DNA complex formed at each time point. The half lives of the DNA–protein complexes were calculated by using the formula $N = N_o e^{-\lambda t}$. λ was obtained from the slopes of the ln[DNA–protein] versus time plots.

RESULTS

E12 homodimers dissociate from κE2 DNA much faster than E47 homodimers

Because of an inhibitory domain present at the N-terminus of E12 basic region, E12 homodimers bind to DNA very poorly as compared with E47 homodimers. This is observed not only in truncated forms of E12 and E47, but also in the full-length proteins. As shown in Figure 1, when *in vitro* translated full-length E12 and E47 were used in the EMSA, specific binding complexes were only detected in the reaction with E47. To further analyze the different DNA binding properties of E12 and E47, we performed filter binding assays to determine the stabilities of DNA complexes formed with E12 and E47 homodimers. The advantage of the filter binding assay is that it allows us to detect unstable DNA–protein complexes. In these experiments, we applied DNA binding reaction onto a nitrocellulose membrane, which retains DNA–protein complexes but not free DNA, and washed the membrane with appropriate volumes of washing buffer using a vacuum device. Figure 2A shows the dissociation curves of κE2 DNA complexes with E47N and E12N, which is obtained by plotting the amount of DNA–protein complex with time after addition of the competitor oligonucleotides. To obtain linear curves, we plotted ln[DNA–protein] versus time based on the formula $N = N_o e^{-\lambda t}$ (Fig. 2B). It is noted that the curve for E12N reaches a plateau after 2 min. We reason that the counts from E12N samples 2 min after addition of the competitor DNA may be contributed by the non-specific DNA binding activity of E12N. Therefore, it is probably appropriate to consider just the linear portion of the curve, albeit contains only three time points. By using the slopes obtained from the linear curves for E12N and E47N, we estimated the half lives of the E12N and E47N complexes to be 0.53 and 6.93 min respectively (a 13-fold difference). Similar values can also be obtained from the curves shown in Figure 2A by estimating the times taken to dissociate 50% of the complexes. These data led us to conclude that the apparently poor binding activity of E12 may be attributed to the fast dissociation of DNA–protein complexes and thus, this unstable E12 complex cannot be easily detected using EMSA. Even by filter binding assays, we reproducibly observe a lower binding activity of E12N in comparison with E47N at time zero. The lower apparent binding activity may be partially due to the fast dissociation rate of the E12 complex. Since the half life of the E12 complex is ~0.5 min, ≥ 50% of the complex formed at time zero is dissociated during the filter binding assay procedure, which takes ~0.7 min from loading the binding reaction onto the filter to the completion of the washing step.

Basic amino acid substitution increases the DNA binding activity of E12 homodimers

It has previously been shown that E12 mutants whose basic region is replaced with that of MyoD or myogenin can bind efficiently to the consensus E-box sequence CAGGTG (16,19) as

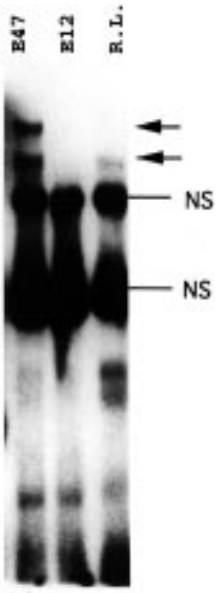


Figure 1. Electrophoretic mobility shift assay of full-length E12 and E47 as indicated. Aliquots (5 µl) of each *in vitro* translation reaction that contains similar amount of E12 or E47 protein were used for the binding reactions. Unprogrammed reticulocyte lysate (R.L.) was used in a binding reaction as a negative control. Non-specific binding complexes are labelled as NS. A 64 bp DNA fragment containing the κE2 site was used as a probe.

homodimers when assayed by EMSA. This observation suggests that sequences in the basic region of MyoD and myogenin enables E12 homodimers to bind to DNA. To identify such a sequence, we have created Mutants 1, 2 and 3, in which parts of E12 sequence are changed into the corresponding sequence in MyoD (Fig. 3). DNA binding activities of these mutants were then analyzed by electrophoretic mobility shift assays using the κE2 site in the intronic enhancer of the immunoglobulin κ light chain gene as a probe. As shown in Figure 4, substitutions in Mutant 1 have little effect on the DNA binding activity, but a single amino acid change from valine to lysine at position 4 (Mutant 2) and leucine to arginine at position 12 (Mutant 3) of the basic region resulted in a dramatic increase of DNA binding activity. By EMSA, Mutants 2 and 3 show as much DNA binding activity as E47 or E12-MyoDBJ, which contains the entire basic region of MyoD substituted for that of E12. Since mutations that replace the two asparagine residues at positions 6 and 7 with alanine and threonine have previously been shown not to alter the DNA binding property (16), we have thus pinpointed the amino acid residues at positions 4 and 12 in the basic region of MyoD that are responsible for increasing the DNA binding activity of E12 homodimers. Evidently, both of these residues are basic amino acids. We were thus prompted to test whether substitution of basic amino acids at other positions in the basic region could have the same effect as those at position 4 and 12. Indeed, when a lysine residue was put in place of the alanine residues at positions 5 and 8 respectively, the DNA binding activity was drastically increased (Figs 3 and 4). It therefore appears that introduction of an additional basic amino acid to the basic region is sufficient to elevate the apparent DNA binding activity of E12 homodimers to the level of E47 homodimers.

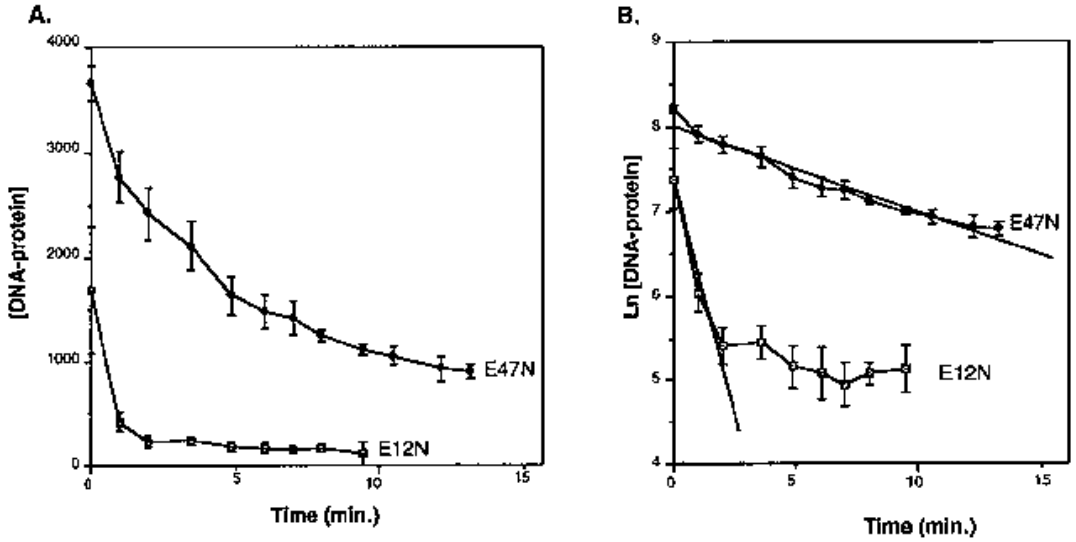


Figure 2. Filter binding assays of E12N and E47N. (**A**) [DNA–protein] versus time plot. (**B**) ln [DNA–protein] versus time plot. Binding reactions were performed with *E.coli* extracts containing overexpressed E12N and E47N. E47N binding reaction (20 µl) and 40 µl E12N reaction were loaded onto the filter. The concentrations of DNA–protein complexes ([DNA–protein]) are represented by the counts retained after washing (in c.p.m.) subtracted from a background count determined with an *E.coli* extract without a κE2 binding protein (70 c.p.m. per 20 µl binding reaction). All experiments were performed in triplicate and the values are the averages with standard deviations. Slopes were obtained from the straight lines shown in (B).

Only basic amino acids can increase the DNA binding activity of E12

We next asked whether only the basic amino acid substitutions could enable E12 homodimers to bind DNA efficiently in the presence of its inhibitory domain. We generated a series of mutants that contain different amino acid at position 4 (Fig. 3) and assayed their abilities to bind to the κE2 probe. To test whether these amino acid substitutions in the basic region would affect the intrinsic DNA binding ability of E12 in the absence of the inhibitory domain, we created a cognate mutant for each amino acid substitution, in which the inhibitory domain was inactivated by a four-amino acid mutation (Fig. 3; 4). As shown in Figure 5, although these mutants can bind to DNA without the inhibitory domain, substitution mutants with glutamic acid, aspartic acid, glutamine, isoleucine or serine cannot bind to DNA efficiently in the presence of the inhibitory domain. A substitution mutant with methionine at this position also has no effect on the DNA binding activity of E12 (4). In contrast, substitution with lysine and arginine at this position results in DNA binding with or without the inhibitory domain. Substitution with a proline residue abolishes DNA binding even in the absence of the inhibitory domain. These results have led us to conclude that only the basic amino acid substitutions can increase the apparent DNA binding activity of E12 homodimers as measured using EMSA. However, the mechanism that causes the increase is not clear.

Basic amino acid substitution increases the intrinsic stability of E12–DNA complexes

It remains to be resolved whether the increase of DNA binding activity by the basic amino acid substitutions is achieved by alleviating the repression from the inhibitory domain of E12 or by increasing the intrinsic stability of E12–DNA complexes. Using filter binding assays, we have found that mutations of the inhibitory domain of E12 (E12N-mI) lead to a great increase of the stability of the DNA binding complexes and that DNA complexes with Mutant 2K is three times more stable than those with E12N (data not shown). Since the inhibitory domain renders E12–DNA complexes unstable, it is conceivable that mutations 2K and 2R increase the stability of binding complexes by counter-reacting with the inhibitory domain. However, it is also possible that 2K and 2R mutations stabilize the complexes independent of the inhibitory domain. To distinguish the two possibilities, we have analyzed the stability of DNA binding complexes of the wild type or mutant E12 proteins with or without the inhibitory domain. The proteins without the inhibitory domain contain point mutations in the inhibitory domain known as mI (E12N-mI, 2K-mI, 2R-mI). We have examined the dissociation rates of these proteins using EMSA, and quantified them by plotting ln [DNA–protein] versus time as shown in Figure 6. The DNA complexes with wild-type E12N homodimers were too unstable to be analyzed by EMSA, but those of E47N homodimers can be measured. Thus, the dissociation rate of E47 complexes measured by EMSA can serve as a reference to the dissociation rates determined by filter binding assays. The half life for E47N determined by EMSA is 2.19 min, while that determined by the filter binding assay is 6.93 min. Therefore, the half live for E12N would be 0.17 min if it was determined by EMSA. Complexes with mutants 2K or 2R are detectable by EMSA but dissociate faster than that with E47 (comparing $t_{1/2}$ = 0.5 min with $t_{1/2}$ = 2.19 min), a finding similar to that obtained using filter binding assays (data not shown). Without the function of the inhibitory domain in the E12N-mI protein, the stability of the DNA binding complexes is increased by ~43-fold ($t_{1/2}$ = 7.29 min). However, substitution of a basic amino acid at position 4 further stabilizes the binding complexes as found in 2K-mI or 2R-mI ($t_{1/2}$ = 20.76). If the basic amino acid substitution increases the stability by counter-reacting with the inhibitory domain, E12N-mI would have had the same dissociation rate as 2K-mI and 2R-mI. It thus appears that these basic amino acid substitutions elevate the intrinsic DNA binding activity of E12 by 3-fold regardless of the inhibitory domain. By doing so, the binding complexes of mutants 2K and 2R become detectable under our EMSA condition. To rule out the possibility

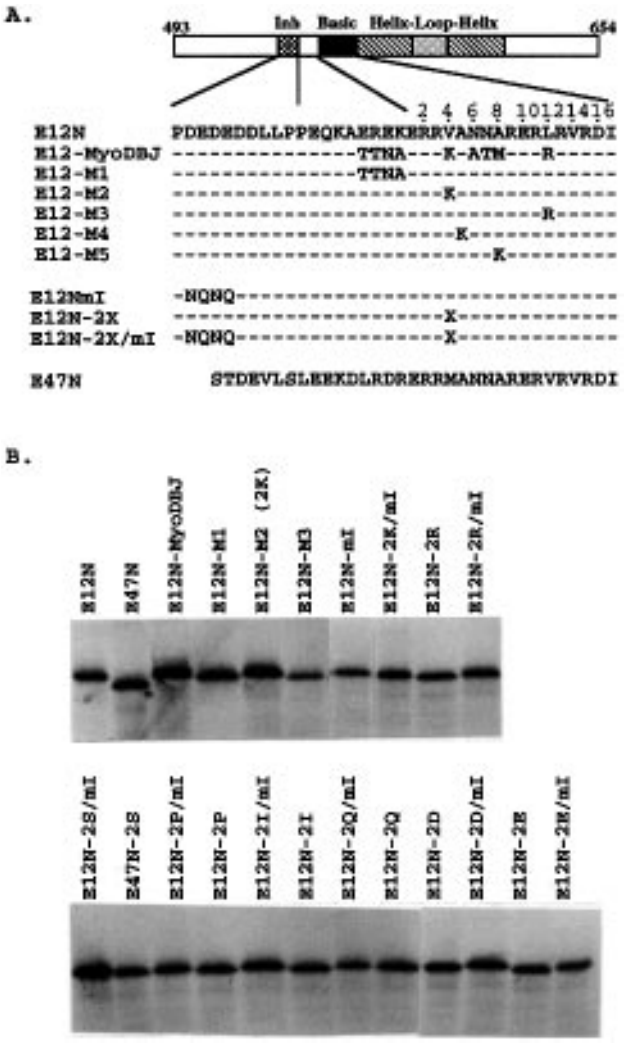


Figure 3. (**A**) Diagrams of E12 mutants. Truncated wild-type and mutant E12 proteins (from amino acid 493–654) are shown as an open bar with the inhibitory domain, basic region and helix–loop–helix motif depicted by crossed, solid and hatched areas. The sequence from the inhibitory domain to the basic region is shown below. A dash (–) in the sequence shows a conserved amino acid while letters represent the corresponding amino acid substitutions that were made. Numbers indicate the position of each residue in the basic region. Names of the mutant proteins are listed on the left. E12N-2X represents mutants that have amino acid substitution at position 4 of the basic region. X stands for K, R, D, E, Q, I, S or P. (**B**) The amounts of ^{35}S-labelled proteins used in the binding assays shown in Figures 4, 5 and 6. E12N-M4 and M5 are not presented here but they have been quantified using PhosphorImager and adjusted to the amount of E12N in the binding reactions.

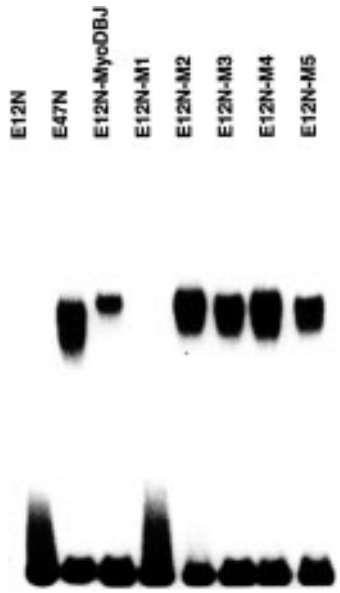


Figure 4. Electrophoretic mobility shift assay of E12 basic region mutants. Equal amounts of E12N E47N, E12N-MyoDBJ and E12N-M1–E12N-M5 were used in the binding reaction and analyzed on a 4% non-denaturing gel.

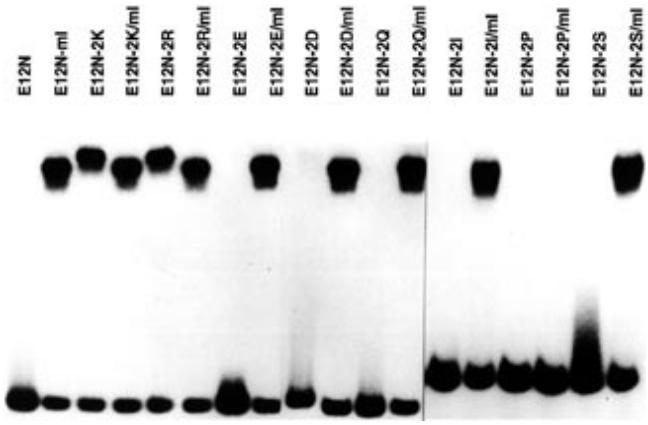


Figure 5. Electrophoretic mobility shift assays of point mutations at position four of the E12 basic region. The odd-numbered lanes contain proteins with wild-type inhibitory domain and the even-numbered lanes contain proteins with mutated inhibitory domain (mI). Equal amounts of E12N, E12N-2K, 2R, 2E, 2D, 2Q, 2I, 2P and 2S, along with their cognate inhibitory domain mutants (mI) were used in EMSA with the κE2 probe.

that mutations in the inhibitory domain (in the E12N-mI protein) can increase the intrinsic stability of the complexes rather than inactivating the inhibitory domain, we have also created N-terminal truncation mutations that delete the entire inhibitory domain in E12N, E12N-2K and E12N-2R, and found these proteins to have similar stabilities as the proteins with the mI mutations (data not shown).

DISCUSSION

This report elucidates the mechanism by which the inhibitory domain of E12 prevents DNA binding, i.e. by de-stabilizing DNA–protein complexes, and the mechanism by which basic amino acid substitution in the basic region facilitates DNA binding, i.e. by stabilizing the DNA–protein complexes. Although the inhibitory domain and the substituted basic amino acids do not appear to interact directly, their negative and positive effects dictate the DNA binding activity of E12 homodimers. From the structural point of view, how are the negative and positive effect achieved? The inhibitory domain of E12 has been proposed, based on previous mutagenesis data, to form a negatively charged helix that creates steric hindrance for the DNA–protein complex (4). We suspect that this hindrance involves interaction with DNA, because we consistently observe that E12 binds to the κE2 site much less efficiently when the site is located in a longer DNA fragment (e.g.

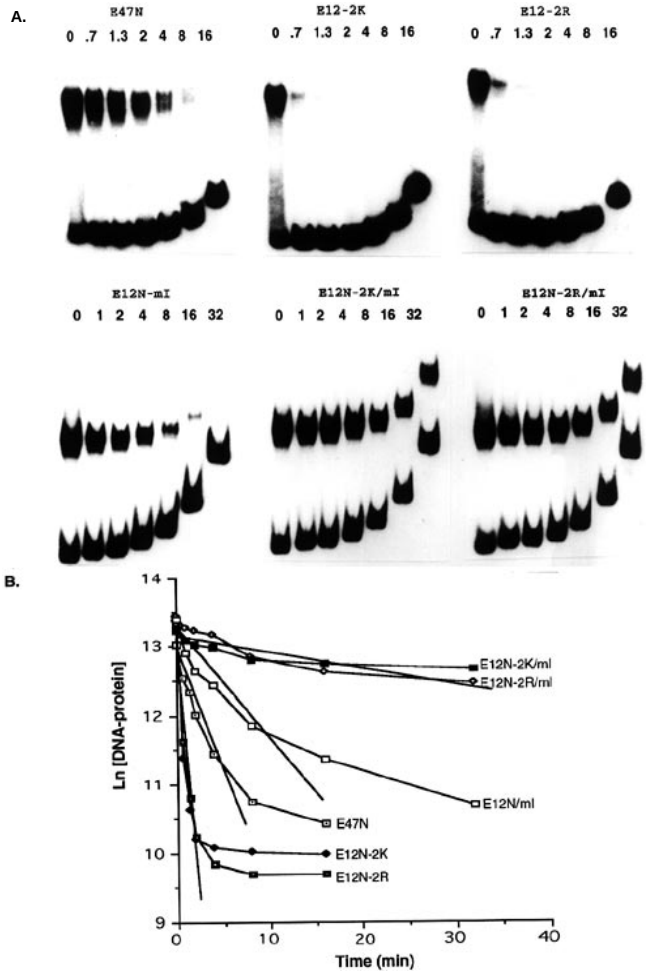


Figure 6. Dissociation rates of E12 and E47 wild-type and mutant proteins. (**A**) The experimental procedure is described in the Material and Methods section. The protein used is indicated on the top of each set of EMSA data. The time point is labelled on top of each lane expressed in minutes. (**B**) The ln[DNA–protein] versus time plot. [DNA–protein] is represented by the counts determined using PhosphorImager. Slopes were obtained from the straight lines for E12N-2R and 2K, E47N, E12N-mI, and E12N-2R-mI and 2K-mI.

64 or 56 bp) than when it is in a 20 bp oligonucleotide (data not shown). When E12 is bound to a larger piece of DNA as they do *in vivo*, the effect from the inhibitory domain becomes apparent, but the effect is alleviated when bound to a short oligonucleotide. According to the crystallographic data of E47 (20), amino acids at positions 4, 5, 8 and 12 in the basic region are not responsible for forming hydrogen bonds with the specific nucleotides. Therefore, basic amino acids at these positions may be available for non-specific interaction with the phosphate backbone through their positively charged amino groups, which result in the stabilization of the DNA–protein complex.

What is the biological significance of the stabilization of DNA–protein complexes by the basic amino acids? One explanation may come from the muscle system. All of the muscle-specific bHLH proteins, MyoD (8), Myogenin (6,7), Myf5 (5) and MRF4 (10), possess basic amino acids at positions 4 and 12. It has been shown that heterodimers of MyoD with E12 or E47 binds to the E-box sequence more efficiently than E12 or even E47 homodimers (4). Since only heterodimers of the myogenic bHLH proteins with the ubiquitous E2A proteins are able to activate muscle-specific gene expression and differentiation (2,19), it is therefore very important to ensure that only these heterodimers not E2A protein homodimers bind to the relevant E-box sites (the myogenic bHLH proteins do not form homodimers efficiently). To compete with the E2A protein homodimers, the heterodimers must have a higher affinity for the binding site. However, this does not mean that E2A protein homodimers are of no use. They have been shown to exist in B lymphocytes (12,21,22). Inactivation of the E2A function blocks B cell development in mice (13–15). Is the DNA binding affinity of the homodimers high enough? In B cells, perhaps only in B cells, gel-shift complexes consisting of E2A homodimers are readily detectable by EMSA (12,21). It remains to be determined whether this is due to a higher level of E2A expression in B cells, or whether B cell specific post-translational modification may occur such that the DNA–protein complexes would be stabilized. Based on our *in vitro* studies, we would also predict that the E2A homodimers detected in B cells are mostly E47 homodimers not E12 homodimers. Super shift experiments with the antibodies presently available do not allow us to directly address this issue. It is therefore extremely interesting to determine whether E12 and E47, when expressed in the E2A deficient mice, can both rescue B cell development or if only E47 can do so.

The E2A gene is transcribed constitutively and ubiquitously to produce mRNAs for both E12 and E47 by differential splicing at the exons that encode the bHLH domain of E12 and E47 (1,4). This raises the question as to what is the selection pressure to preserve both E12 and E47 exons for the DNA binding and dimerization motifs, which are evolutionarily conserved from Xenopus to man (1,23–25). Although the answer would ultimately come from experimental data, one might speculate that it is important to have the different DNA binding properties of E12 and E47 proteins. Both E12 and E47 form heterodimers with tissue-specific bHLH transcription factors and bind to the κE2 site efficiently. Both proteins can dimerize with Id proteins at a similar efficiency and therefore be inhibited by them (26). E47 also binds DNA as homodimers, but E12 binds very poorly. We have shown here that this poor binding activity of E12 is primarily due to a fast dissociation of E12 from DNA. This difference in the dissociation rates between E12 and E47 might be necessary for the two proteins to perform their different functions. In certain situations, it might be important to have E47 bound to DNA stably to maintain an open complex to activate transcription. The transient binding of E12, on the other hand, is probably appropriate for reserving a pool of free E2A proteins for regulated transcription of certain genes. For example, when E12 homodimers bind to a κE2 site next to another transcription factor which could stabilize E12 binding, the two proteins would synergistically activate the transcription of a specific gene. In the absence of the second transcription factor, E12 would dissociate immediately from a κE2 site that might not be of any regulatory importance. In this way, additional specificity of the κE2 site as a control element may be acquired.

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REFERENCES

1 Murre,C., McCaw,P.S. and Baltimore,D. (1989) *Cell*, **56**, 777–783.
2 Davis,R.L., Cheng,P.F., Lassar,A.B. and Weintraub,H. (1990) *Cell*, **60**, 733–746.
3 Voronova,A. and Baltimore,D. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4722–4726.
4 Sun,X.-H. and Baltimore,D. (1991) *Cell*, **64**, 459–470.
5 Braun,T., Buschhausen,D.G., Bober,E., Tannich,E. and Arnold,H.H. (1989) *EMBO J.*, **8**, 701–709.
6 Brennan,T.J. and Olson,E.N. (1990) *Gene Dev.*, **4**, 582–595.
7 Wright,W.E., Sassoon,D.A. and Lin,V.K. (1989) *Cell*, **56**, 607–617.
8 Davis,R.L., Weintraub,H. and Lassar,A.B. (1987) *Cell*, **51**, 987–1000.
9 Murre,C., McCaw,P.S.,Vaessin,H., Caudy,M., Jan,L.Y., Jan,Y.N., Cabrera,C.V., Buskin,J.N., Hauschka,S.D., Lassar,A.B., Weintraub,H. and Baltimore,D. (1989) *Cell*, **58**, 537–544.
10 Rhodes,S.J. and Konieczny,S.F. (1989) *Genes Dev.*, **3**, 2050–2061.
11 Johnson,J.E., Birren,S.J. and Anderson,D.J. (1990) *Nature*, **346**, 858–861.
12 Bain,G., Gruenwald,S. and Murre,C. (1993) *Mol. Cell. Biol.*, **12**, 3522–3529.
13 Zhuang,Y., Soriano,P. and Weintraub,H. (1994) *Cell*, **79**, 875–884.
14 Bain,G., Maandag,E.C.R., Izon,D.J., Amsen,D., Kruisbeek,A.M., Weintraub,B.C., Krop,I., Schlissel,M.S., Feeney,A.J., van Roon,M., van der Valk,M., Riele,H.P.J., Berns,A. and Murre,C. (1994). *Cell*, **79**, 885–892.
15 Sun,X.-H. (1994). *Cell*, **79**, 893–900.
16 Davis,R.L. and Weintraub,H. (1992) *Science*, **256**, 1027–1030.
17 Studier,F.W. and Moffatt,B.A. (1986) *J. Mol. Biol.*, **189**, 113–130.
18 Ausubel,F.M., Brent,R. Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) 1987, *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
19 Chakraborty,T., Brennan,T.J., Li,L., Edmondson,D. and Olson,E.N. (1991) *Mol. Cell. Biol.*, **11**, 3633–3641.
20 Ellenberger,T., Fass,D., Arnaud,M. and Harrison,S.C. (1994) *Genes Dev.*, **8**, 970–980.
21 Jacobs,Y., Vierra,C. and Nelson,C. (1993) *Mol. Cell. Biol.*, **13**, 7321–7333.
22 Xin,X.Q., Nelson,C., Collins L. and Dorshkind,K. (1993) *J. Immunol.*, **151**, 5398–5407.
23 Aronheim,A., Shiran,R., Rosen,A. and Walker,M.D. (1993) *Nucleic Acids Res.*, **21**, 1601–1606.
24 Nelson,C., Shen,L.P., Meister,A., Fodor,E. and Rutter,W.J. (1990) *Genes Dev.*, **4**, 1035–1043.
25 Rashbass J., Taylor,M.V. and Gurdon,J.B. (1992) *EMBO J.*, **11**, 2981–2990.
26 Sun,X.-H., Copeland,N.G., Jenkins,N.A. and Baltimore,D. (1991) *Mol. Cell. Biol.*, **11**, 5603–5611.