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Derivation of the consensus DNA-binding sequence for p63 reveals unique requirements that are distinct from p53

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Abstract **p63 is a member of the p53 family of proteins and plays an important role in epithelial development and differentiation. Although some p63 binding sites in the regulatory elements of epithelial genes have been identified, the optimal DNA-binding sequence has not been ascertained for this transcription factor. Here, we identify the preferred DNA-binding site of p63 by performing in vitro DNA selection experiments. Our analysis shows that the optimal p63 DNA-binding consensus motif consists of a CA(T)TG core and an AT-rich 5′ and 3′ flanking sequence. Gel shift and competition experiments demonstrate that there are specific sequence requirements that confer high DNA-binding affinity for p63 and that significant deviation from the consensus sequences result in poor or no binding. This pattern of DNA-binding is similar for both recombinant p63 and the endogenous protein present in keratinocyte nuclear extracts. Furthermore, we show that the consensus sequence is distinctly different from that of p53, particularly in the flanking sequences. Identification of the p63 consensus DNA-binding sequence will facilitate the validation of in vivo p63-responsive elements that mediate transcriptional regulation of a wide variety of target genes.**

Keywords: p63; Systematic evolution of ligands by exponential enrichment; Electrophoretic mobility shift assays; Keratinocyte

1. Introduction

p63 is a member of the p53 tumor suppressor gene family that plays a crucial role in transcriptional regulation of a number of genes involved in developmental and cellular responses [1]. In particular, p63 is involved in limb, skin and craniofacial morphogenesis as evident by the dramatic phenotype of the p63 null mice [2,3]. These animals exhibit profound defects in the skin epidermis as well as abnormalities of limb, mammary and prostate development. In addition, germ line mutations of p63 in humans are associated with congenital abnormalities characterized by abnormal limb development and/or ectodermal dysplasia, phenotypes that are similar to p63 null mice [4–6]. The physiological role of p63 in the epithelial development and differentiation process is in agreement with high levels of expression of p63 in select epithelial cells such as those present in the embryonic ectoderm and the basal proliferating cells of many epithelial tissues such as skin, oral epithelium, breast myoepithelium and prostate [4,7–9].

The p63 gene is complex and encodes for at least six unique isoforms. Differential promoter usage produces two major protein isoforms that either contain or lack the N-terminal transactivation domain (TAp63 and ΔNp63, respectively). In addition, differential mRNA splicing leads to generation of three distinct isoforms (α, β and γ), which differ in their C-terminal domains. This proclivity to generate multiple isoforms is not restricted to p63 alone, but is also characteristic of p73, another p53 family member. As recent studies indicate, this is a feature shared by p53 as well [10]. Structurally, the p63 protein is organized similar to p53 and p73 and consists of an N-terminal activation domain, DNA-binding domain in the middle and an oligomerization domain towards the C-terminus. As expected, the highest homology between the p53 family members is found in the DNA-binding domain. However, an approximately 60% homology within the DNA-binding domain between these proteins is relatively modest compared to other families of transcription factors such as AP-2 which share up to 90% homology [1,11].

Transcriptionally active forms of p63 are likely to regulate target gene expression by sequence-specific recognition of control response elements present in promoters and enhancers. Because both p53 and p63 contain similar DNA-binding domains, it has been thought that the p63-response elements are likely to be similar to that of the canonical p53 response element. Indeed, several studies have shown that p63 can bind to p53-response elements and activate transcription of p53-inducible genes such as MDM2, p21, Bax and 14-3-3σ [4]. However, increasingly it is becoming clear that unique p63 target genes exist, as would be expected from the fact that the in vivo function of p63 is strikingly different from p53.

One possible mechanism by which p63 can regulate unique target genes is by preferentially binding to and activating distinct response elements. In this case, the selectivity of p63 transcription factor for a specific promoter and/or enhancer will depend to a large extent on the nature of the DNA-binding site. However, to this date the nature of such p63-binding sites has not been experimentally determined. Here we have performed systematic evolution of ligands by exponential enrichment (SELEX) experiments to decipher the optimum DNA-binding sequence of p63. Our studies show that optimum DNA-binding by p63 requires a CA(T)TG core and an AT-rich 5′ and 3′ flanking sequence. Interestingly, a systematic biochemical analysis of the DNA consensus sequence by direct

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gel shift analysis reveal that DNA-binding of p63 protein requires not only the specific core sequence, but that the flanking sequences also influence the affinity of the protein to DNA. This binding pattern is similar for both recombinant and native p63 and is distinct from that of p53 suggesting that in vivo p63 may indeed bind to unique DNA sequences. The wide range of DNA-binding affinity of p63 may play an important role in mediating its effect on target gene expression.

2. Materials and methods

2.1. Systematic evolution of ligands by exponential enrichment (SELEX)

A 80-bp oligonucleotide was synthesized (IDT Technologies), which contained 35-base random nucleotides flanked by sequences complementary to primers A and B for cloning purposes. The sequences of these three oligonucleotides are as follows: 80-base oligonucleotide, 5′-GGATCCCTGCCTTCACCGAAGC $(N)_{35}$ TTGGGGACTATGAATTCCTGAGG-3′; primer A, 5′-GGATCCCTGCCTTCACCGAAG-3′; primer B, 5′-CCTCAGGAATTCATAGTCCCC-3′. A random sequence library of double-stranded radiolabeled oligonucleotides was prepared by annealing the 80-base oligonucleotide to 5-fold molar excess of primer B followed by extension with Klenow.

The labeling reaction mixture contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM each of dATP, dGTP and dTTP and was incubated with 5 U of the Klenow enzyme at 37 °C for 1 h in the presence of [α-^{32}P] dCTP. The radiolabeled probes were purified by using G-50 Nick columns (Amersham). Binding reactions were performed by adding 100 ng of recombinant His-tagged ΔNp63 protein to DNA binding buffer, DBB (5% glycerol, 10 mM HEPES, pH 7.9, 75 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 1 mM EDTA) containing 0.1 μg of poly (dA · dT) poly(dA · dT) and 5 μg of bovine serum albumin. After incubation at room temperature for 30 min, the protein–DNA complexes were resolved by electrophoresis through a non-denaturing 5% polyacrylamide gel in 1× Tris–borate–EDTA (TBE) buffer at 100 V for 3 h. The complexes specifically formed in the presence of p63 proteins were detected by autoradiography, excised from gels, and eluted overnight at 65 °C in DNA-elution buffer containing 0.3 M NaCl, 1 mM EDTA, and 0.1% SDS. The eluted DNA was extracted once with phenol–chloroform, and then precipitated with ethanol. Primers A and B were used to PCR amplify the purified DNA in the presence of [α-^{32}P] dCTP. Amplification was carried out by 20 cycles of denaturation at 94 °C for 20 s, annealing at 49 °C for 20 s, and extension at 72 °C for 30 s. The amplified DNA was purified using G-50 Nick columns, and used in subsequent EMSA experiments. After four cycles of SELEX, the final amplified DNA was directly cloned using pCR2.1-Topo TA Cloning kit (Invitrogen). Nucleotide sequences of 98 independent clones were determined. The degenerate portion of the sequences was compiled and analyzed for shared sequence patterns by visual inspection and with matrix-based pattern and motif discovery programs such as MEME and CONSENSUS.

2.2. Plasmid constructions

The cDNAs corresponding to mouse ΔNp63α and ΔNp63γ were cloned into the *Bam*HI and *Hin*dIII restriction enzyme sites of the pCOLD vector (Takara). The cDNAs were obtained by searching the expressed sequence tag (EST) database and were modified by PCR strategy for creating in-frame fusion with the His-tag. A plasmid for bacterial expression of His-tagged p53 protein was a gift from Dr. Hua Lu. DNA sequencing of all the constructs was performed to ensure proper reading frame and the absence of any mistakes during PCR amplification.

2.3. Expression and purification of His-tagged proteins

The production and purification of the His-tagged p53 and p63 proteins from bacteria were performed according to the standard protocols. Briefly, cells were centrifuged, and the pellets were resuspended in a buffer containing 50 mM Tris–Cl (pH 8.5), 100 mM NaCl, 1 mg/ml lysozyme, 1 mM PMSF, 1% sarcosyl, and 1% Triton-X-100. The cells were lysed by sonication followed by centrifugation of the lysate at 8500 × *g* for 30 min. The supernatant was mixed with Ni-charged His-Bind resin (Novagen) and the mixture was agitated for 30 min at room temperature. The mixture was then centrifuged at 400 × *g* for 1 min and the supernatant was discarded. The His-bind resin was washed thoroughly first with binding buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidizole, pH 7.9) followed by wash buffer (0.5 M NaCl, 60 mM imidizole and 20 mM Tris–HCl, pH 7.9). The recombinant proteins were eluted in a buffer containing 1 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl, pH 7.9. The quality and quantity of the proteins were verified by SDS–PAGE followed by Coomassie brilliant blue staining. Western blots were performed to address the anti-p53 and anti-p63 antibodies specificity.

2.4. Electrophoretic mobility shift assays with His-p63, His-p53 and keratinocyte nuclear extracts

Electrophoretic mobility shift assays (EMSAs) were performed with either 100–300 ng of recombinant proteins or 5 μg of nuclear extracts and end-labeled double-stranded oligonucleotides. Complementary oligonucleotides were synthesized (IDT Technologies), annealed, and 2 pmole of double-stranded oligonucleotides were used for radioactive labeling with [α-^{32}P] dCTP by Klenow enzyme. The labeled probes were purified by using G-50 Nick columns. Binding reactions were performed at room temperature for 30 min in 20 μl of DBB. 0.1–0.5 μg of poly (dA · dT) poly(dA · dT) was added to each reaction as a nonspecific DNA competitor. Competition assays were performed by incubating recombinant proteins with 20-, 200-, or 400-fold excess unlabeled oligonucleotides for 20 min before addition of radiolabeled probe. The protein–DNA complexes were resolved by gel electrophoresis on 4% non-denaturing polyacrylamide gels in 1× TBE buffer at room temperature. After electrophoresis, the gels were dried and visualized by autoradiography. Anti-p63 and anti-Sp1 antibodies used for supershift experiments have been previously described [12]. Anti-p53 antibodies were obtained from Santa Cruz (DO-1, sc-126).

2.5. Cell culture and preparation of nuclear extracts

HaCaT cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin. Mouse keratinocytes (MK) were grown in a low Ca^{2+} medium comprised of a 3:1 mixture of Ham's F12 and DMEM supplemented with 15% chelated FBS. Nuclear extracts from HaCaT and MK cells were prepared by standard methods as described before [12].

3. Results and discussion

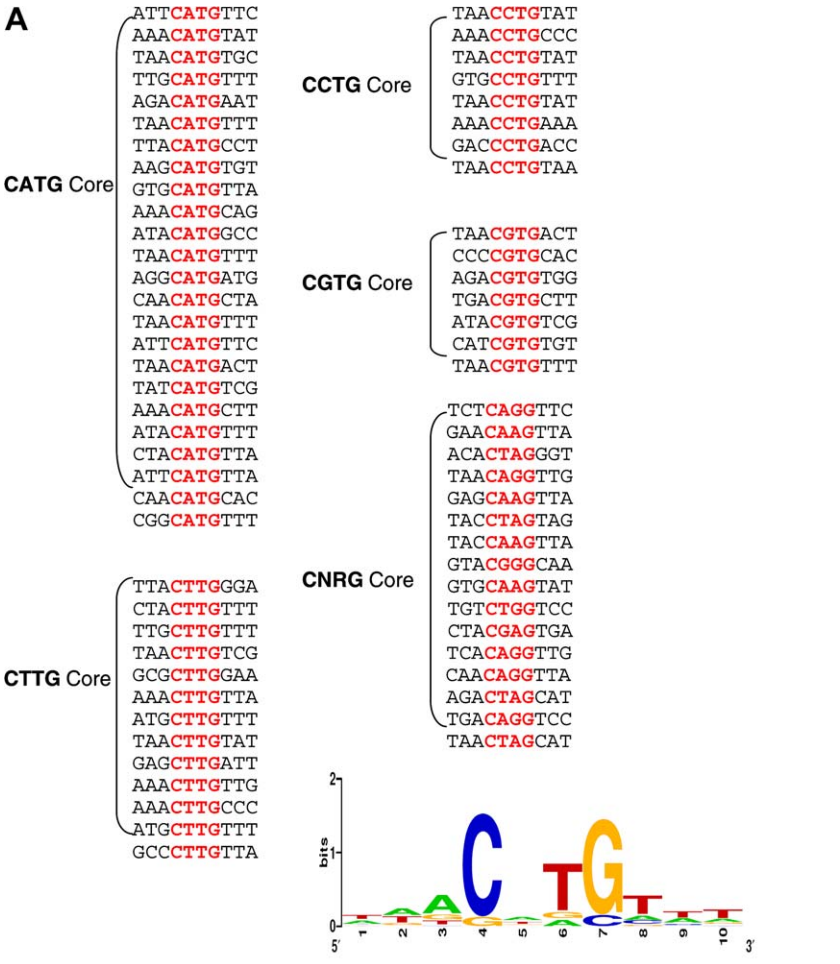
3.1. Selection of p63 consensus DNA-binding sequences

The p53 family of proteins operates as transcription factors, that binds specific DNA sequences and this activity is mediated by the conserved DNA-binding domain. The canonical p53-responsive element has been well described and typically consists of tandem repeats of the sequence, RRRCWWGYYY, where R, purine, C, cytosine, W, adenine or thymidine, G, guanine and Y, pyrimidine. This sequence is commonly repeated as two pairs, where each of the 10 bp palindromic sequences are arranged as inverted repeats with a spacer in between consisting of nucleotides that vary from 0 to approximately 14 bp. Not surprisingly, p63 proteins have been shown to bind to p53 consensus DNA binding sites both in vitro and in vivo. However, the modest sequence similarity in the DNA-binding domain of p53 and p63, the presence of unique targets for these proteins, and their divergent biological role suggest that there may be unique DNA-binding sequence requirements for p63. As a first step towards understanding these DNA sequence requirements for p63, we performed the selection of p63 binding sequences in vitro using the SELEX method [13]. For this purpose, we generated a radioactively labeled pool of double-stranded oligonucleotides containing 35 bp of random sequences in the center flanked by primer sequences. This DNA pool was incubated with recombinant His-ΔNp63α

and the protein–DNA complexes were then separated on a native gel by EMSA. The p63-bound DNA was subsequently recovered from the gel and subjected to 15–20 cycles of PCR amplification. With each round of such selection, an enrichment of specific binding sites was obtained as observed by an increased efficiency of p63–DNA complex formation (data not shown). After five such rounds of selection, the final PCR products were cloned into pCR2.1Topo vector and 98 independent clones were sequenced.

3.2. Alignment of the selected p63 consensus DNA-binding sequences

The sequences were analyzed by visual inspection as well as aligned by using motif search programs such as CONSENSUS and MEME [14,15]. Specifically, we searched for potential p53-like DNA-binding sequences, consisting of the CWWG core. Of the 98 numbers of sequences that were examined, a majority of them had varying degrees of match with the consensus p53–DNA binding sequence. We have categorized these sites into five groups based on the nature of the 4 bp core sequence CNNG. The most common core consisted of CATG sequence, whereas other cores containing CGTG, CTTG and CCTG were present in fewer numbers. Another group of 16 sequences were grouped separately, consisting of CNRG (where R = A/G). The alignment of the p63-binding sequences is shown and the consensus motif depicting the nucleotide distribution that we obtained based on the aligned sequences is represented graphically as a sequence logo (Fig. 1A). For the sake



B

Position	1	2	3	4	5	6	7	8	9	10
% A	37	43	60	0	42	11	0	16	35	23
% C	12	6	5	91	11	1	11	15	14	10
% G	12	15	19	9	16	12	89	7	9	16
% T	38	36	16	0	31	75	0	62	43	51
Consensus	T(A)	A(T)	A	C	A(T)	T	G	T	T(A)	T

Fig. 1. Identification of p63 consensus binding site derived by SELEX. (A) Alignment of the individual sequences generated by the motif search program CONSENSUS. Shown is the alignment of sequences by the position of one of five common core sequences: CATG, CTTG, CCTG, CGTG, CNRG. The consensus sequence is represented graphically as a sequence logo on the bottom. (B) p63 binding site preference based on the sequence of 98 independent clones derived from SELEX. The residues are based on the core sequence with the core cytosine designated as position "4" and core guanine as "7". For each position, the frequency of each nucleotide is indicated as a percentage. The following criteria was used to illustrate the p63 consensus DNA-binding sequence: any nucleotide that is preferred over 50% is shown, if no nucleotide is preferred for more than 50%, then a double degenerate symbol representing the two nucleotides that are the most preferred are shown.

of clarity, we have numbered the 10-bp consensus sequence with the core cytosine being designated as position "4" and core guanine as "7". What is clearly evident from these data is the fact that while there is clearly a preference for C at position 4, T for position 6 and G for position 7, the rest of the positions exhibit less stringent preference for a single nucleotide. Nevertheless, analysis of the sequences suggests that while on the 5′ end of the core sequence there is a preference for an A nucleotide, on the 3′ end of the core, there is a significant preference for T. Taken together, our experiments define the optimal p63 DNA-binding consensus motif as (T/A)A(T)A-CA(T)TGT(T/A)T consisting of a CA(T)TG core and AT-rich 5′ and 3′ flanking sequences (Fig. 1B). Interestingly, none of the sequences contained more than one copy of the consensus site, which may be because of the limitation of the length of the random sequences present within the synthetic oligonucleotide. Alternately, it is possible that p63 may not have a particular preference for tandem repeat sequences for optimum DNA binding.

3.3. Analysis of selected p63 binding sequences by EMSA

The fact that the p63 consensus site consisted of a CA(T)TG core sequence and a preference for A/T rich flanking sequences, prompted us to examine experimentally the sequence requirements in more detail. Specifically, we examined the affinity of p63 to the various core sequences and the role of the 5′ and 3′ flanking sequences. To facilitate these studies, we divided the compiled sequences into four groups, each group representing four different core sequences (Table 1). Thus, Group 1 consists of the core sequence CATG, Group 2 consisted of core sequence CTTG, Group 3 consisted of core sequence CCTG, and Group 4 consisted of core sequence CGTG. Within each group we chose four representative sequences, one that matches perfectly with the p63-consensus sequence, one that contains the core and a perfect match only on the 5′ flanking sequence, one that contains the core and a perfect match only on the 3′ flanking sequence and a fourth that only has a match with the core, while the flanking sequences are highly divergent from the consensus. In each case, the mismatch sequences of the 5′ and 3′ flanking consist of nucleotides which showed the lowest frequency at each position based on the weight matrix. All these representative sequences were picked from the clones that have been described in Fig. 1. We next generated 16 oligonucleotide probes that were roughly the same number of base pairs and contained these p63 recognition sequences in the center. These probes were labeled to approximately the same level of specific activity and tested by EMSA with His-ΔNp63α. As shown in Fig. 2A, the EMSA data revealed several interesting findings. Oligonucleotides 1–4 corresponding to Group 1 (core sequence CATG) bound to p63 to a varying degree. While oligonucleotide 1 bound well to p63, oligonucleotide 2 containing a mismatch in the 5′ of the core sequence did not bind well (lanes 1 and 2). However, a mismatch on the 3′ end did not affect DNA-binding as illustrated by oligonucleotide 3 (lane 3). On the other hand, for group 2 sequences (core sequence CTTG), oligonucleotide 5 with no mismatch on either side and oligonucleotide 6 with mismatch on the 5′ side were capable of binding to p63 quite well (lanes 5 and 6). Both oligonucleotide 7 with mismatch on the 3′ side and oligonucleotide 8 with mismatch on both sides bound to p63 very poorly (lanes 7 and 8). Oligonucleotides 9–12 corresponding to group 3 containing CCTG core tolerated deviations from the consensus sequence on either side quite well, however showed no binding to p63 when both sides were mismatched (lanes 9–12). Finally, group 4 sequences representing the CGTG core sequences showed efficient binding to p63 only when the match to the consensus was on both sides of the core (lanes 13–16). Thus for each core, several oligonucleotides that deviated from the consensus sequences in the 5′ end, 3′ end or from both sides exhibited moderate or weak binding. In addition, although an A or a T was the preferred nucleotide for position 5 in the core, p63 could also tolerate C and G as illustrated by the DNA-binding of group 3 and 4 oligonucleotides. Collectively, our data suggests that oligonucleotides that more closely resemble the p63 consensus sequence show stronger affinity for p63 and that not only the core but also the flanking sequences play an important role in governing the binding of p63 to DNA.

Table 1
p63 binding sites divided into four groups based on core sequence (in bold)

	Oligonucleotide	Sequence	Binding affinity	Competes?
Group 1	1	GGA ATAAGTAT TAA**CATG**TTT AGAATAGG	++++	Yes
	2	GGA ATAAGTAT ATA**CATG**GCC AGAATAGG	++	Weakly
	3	GGA ATAAGTAT CGG**CATG**TTT AGAATAGG	++++	Yes
	4	GGA ATAAGTAT AGG**CATG**ATG AGAATAGG	+++	Yes
Group 2	5	GGA ATAAGTAT TAA**CTTG**TAT AGAATAGG	++++	Yes
	6	GGA ATAAGTAT AAA**CTTG**CCC AGAATAGG	++++	Yes
	7	GGA ATAAGTAT GCC**CTTG**TTA AGAATAGG	+/−	Weakly
	8	GGA ATAAGTAT GAG**CTTG**ATT AGAATAGG	+	Yes
Group 3	9	GGA ATAAGTAT TAA**CCTG**TAT AGAATAGG	++++	Yes
	10	GGA ATAAGTAT AAA**CCTG**CCC AGAATAGG	++++	Yes
	11	GGA ATAAGTAT GTG**CCTG**TTT AGAATAGG	+++	Yes
	12	GGA ATAAGTAT GAC**CCTG**ACC AGAATAGG	−	No
Group 4	13	GGA ATAAGTAT TAA**CGTG**TAT AGAATAGG	++++	Yes
	14	GGA ATAAGTAT TAA**CGTG**ACT AGAATAGG	+	Weakly
	15	GGA ATAAGTAT TGT**CGTG**TTT AGAATAGG	+	No
	16	GGA ATAAGTAT CCC**CGTG**CAC AGAATAGG	−	No

Within each group 4 oligonucleotides represent sequences which were chosen based on match with flanking sequence.

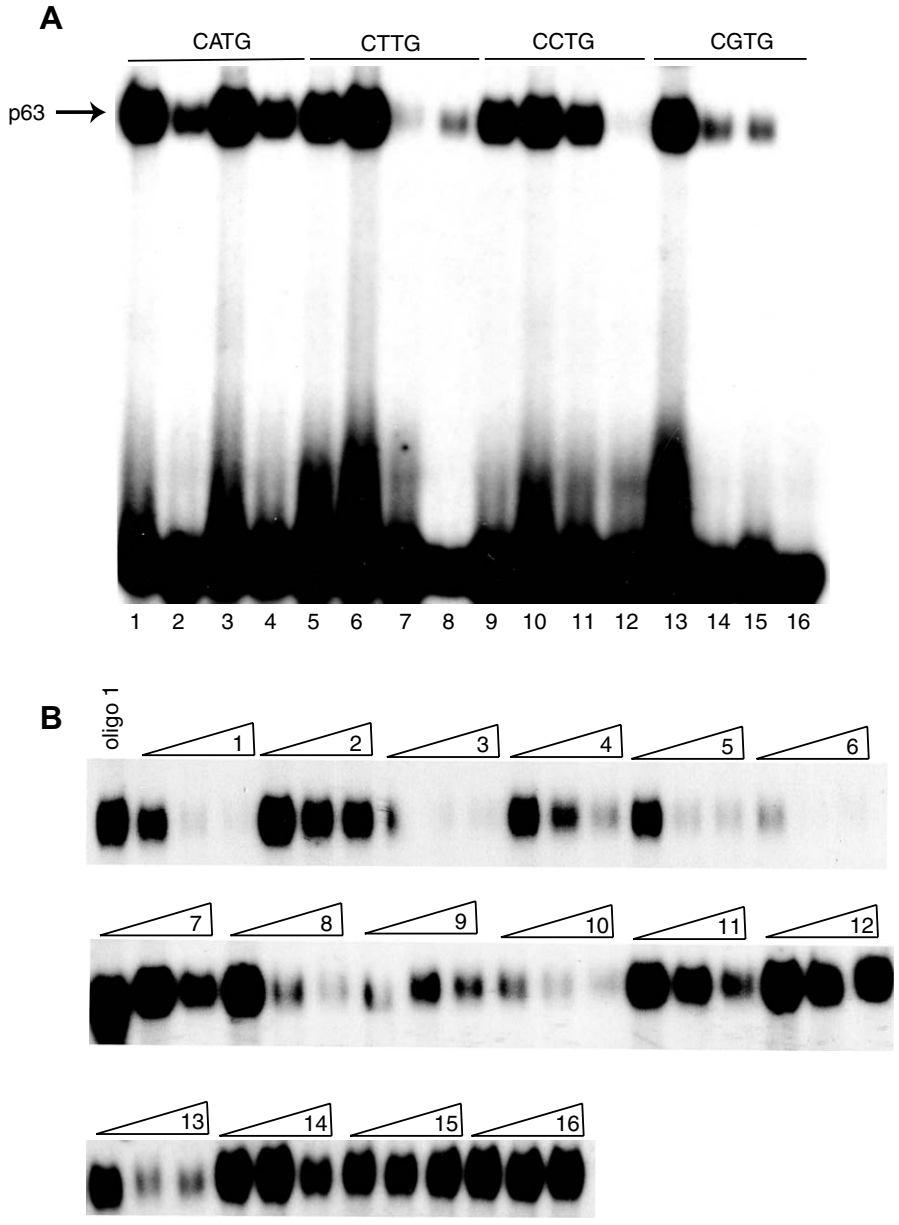


Fig. 2. EMSA reveals the binding specificity of p63 to 16 different oligonucleotides with different degrees of match with the consensus sequence. Numbers on the bottom of each lane indicate the specific oligonucleotides (listed in Table 1) used in this experiment. (A) EMSA showing DNA binding of ΔNp63α to four groups of selected oligonucleotides. (B) Competition for p63 binding by four groups of the selected binding sites. Oligonucleotide 1 was radiolabeled and 20-, 200-, and 400-fold excess amount of the 16 different unlabeled oligonucleotides as indicated above each lane were added to the binding reaction for competition analysis. Only the DNA–protein complexes are shown.

In addition to testing the direct binding of the various oligonucleotides to ΔNp63α, we also confirmed the affinity of these sequences to ΔNp63α by performing competition experiments (Fig. 2B). For this purpose, the oligonucleotides corresponding to sequence 1 were radiolabeled and 20-, 200- and 400-fold excess amount of the 16 different cold oligonucleotides was used to compete the binding of ΔNp63α. The competition experiments validated the data obtained from direct binding, since the degree of competition was as expected from the direct binding studies from Fig. 2A, with strong competition by sequences that matched with the p63 consensus sequences and progressively weaker competition with sequences that diverged from p63 consensus sequences. These relative binding affinities of the different sequences are summarized in Table 1.

Since the p63 gene encodes for a variety of isoforms, one possibility is that p63 isoforms may differentially bind DNA. Indeed, some recent studies suggest that this might be the case as is evident for one recently described p63 target gene, GPX3, which contains a p63-responsive element that is specifically responsive to p63γ and not p63α [16]. To test for different DNA-binding properties of p63 isoforms, we generated His-ΔNp63γ and repeated the EMSA studies with previously described 16 different oligonucleotide probes that represented various degrees of match with the p63 consensus sequence. The pattern of DNA-binding for ΔNp63γ was indistinguishable

from that of ΔNp63α (data not shown). This suggests that at least in vitro, two major isoforms of p63 show no significant difference in DNA binding as expected from the fact that they both share an identical DNA-binding domain. However, in vivo it is possible that additional events such as post-translational modifications and protein–protein interactions may impart some degree of specificity to various p63 isoforms.

3.4. Comparison of the DNA-binding specificity of p63 with endogenous p63

It is possible that the binding activity of the recombinant p63 proteins that were purified from bacteria may not reflect the binding properties of native protein. This could be due to many reasons such as improper folding, non-physiological amounts of the protein and lack of potential post-translational modifications. To test if the binding properties that we observe with His-p63 are similar to that of endogenous p63, we utilized two different cell lines, HaCaT and MK. HaCaT keratinocytes contain mutant p53, high levels of p63 and exhibit complex interactions between p53 and p63 [17]. Since recombinant proteins used in this study were generated with mouse p63 cDNAs, a mouse keratinocyte cell line, MK was utilized to account for any potential species difference. Nuclear extracts from both HaCaT and MK cells were tested with the 16 oligonucleotides containing the various core and flanking sequences as well as an oligonucleotide containing the p53-consensus sequence (Fig. 3A and B). Incubation of the p53-consensus oligonucleotide with nuclear extract resulted in a slow-moving complex, which could be specifically supershifted with antibodies against p63 but not with antibodies against p53 or Sp1. This confirmed that this complex consists of endogenous p63. The antibodies against p53 and p63 were specific and did not cross react (Supplementary Fig. 1). Interestingly, native p63 from both human and mouse bound to the 16 oligonucleotides in a fashion very similar to the recombinant protein. Each of the DNA–protein complexes was formed by p63 as evident by the migration pattern and the fact that each complex reacted with antibodies against p63 (data not shown). These data indicate that the consensus sequence information obtained from our in vitro studies may be useful in assessing the DNA-binding affinity of the endogenous p63 proteins.

3.5. Comparison of the DNA-binding specificity of p63 with p53

As shown by several studies the typical p53-response element is a palindrome sequence (RRRCWWGYYY) [18,19]. Interestingly, p53 binds preferably to the CATG core sequence, suggesting that p53 binds more efficiently to structurally perfect bidirectional palindromes. In contrast, a recent study has shown that p63 has much higher specificity for the CGTG (non-palindromic) core sequence than p53 as suggested by reporter analysis of mutated response elements and by DNA–protein binding assays [20]. However, our in vitro data seems to indicate that the preferred binding site for p63 binding sites contains the CATG core since it is the most common core motif isolated from the random library of sequences and it tolerates mismatches on the 5′ and 3′ side very well. The difference between these results could be because of the fact that the previous studies were performed with TAp63 proteins. Nevertheless, what is evident from these studies is that there is a great deal of heterogeneity of DNA-binding sites for p53 and p63 proteins, which may reflect a difference in their DNA-binding properties. To analyze this further experimentally, we compared the DNA-binding properties of p53 and p63 by performing EMSA with recombinant proteins. We utilized the 16 oligonucleotides that represented the four different groups of p63 binding sites that were derived from the SELEX experiments described before. Interestingly, His-p53 showed different degrees of DNA-binding activity with the 16 oligonucleotides, when compared to p63 (Fig. 4). Indeed, p53 DNA-binding was more promiscuous, since it tolerated a wide variety of mismatches compared to p63. The most striking difference was observed with oligonucleotides 7, 12 and 16, which showed significantly better binding to p53 than to p63. Taken together, our data suggest that although these differences in affinity for specific DNA-binding sites of p63 and p53 are subtle, they may have important implications for target gene specificity in vivo.

To date only about 15 genes have been shown to contain DNA-responsive elements that bind to p63 protein and are transcriptionally regulated by it. In light of our newly derived p63 consensus sequence, we examined these binding sites of p63 as described in the literature to see if they bear resemblance to the consensus. Interestingly many of them matched our derived consensus sequence very well with very few mismatches (see supplementary Table 1). For some of the target

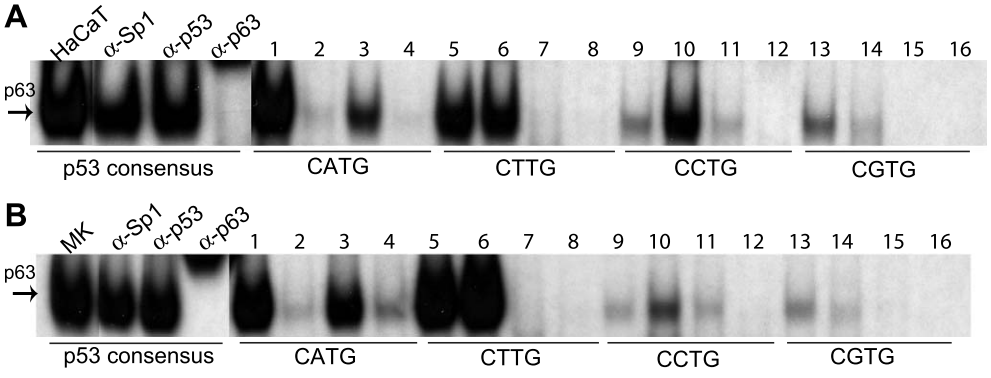


Fig. 3. DNA-binding specificity of endogenous p63 is similar to that of recombinant protein. Nuclear extracts from HaCaT cells (A) and MK (B) were tested with the 16 oligonucleotides as well as an oligonucleotide containing the p53-consensus sequence. Numbers refer to the specific oligonucleotides used in this experiment and the sequences can be found in Table 1. The p53-consensus sequence used for the EMSA is 5′-GGTTACAGAACATGTCTAAGCATGCTGGGG-3′. While antibodies directed against p53 and Sp1 had no effect, anti-p63 antibodies resulted in a super-shift of the complex, indicating that the complex is p63. Only the DNA–protein complexes are shown.

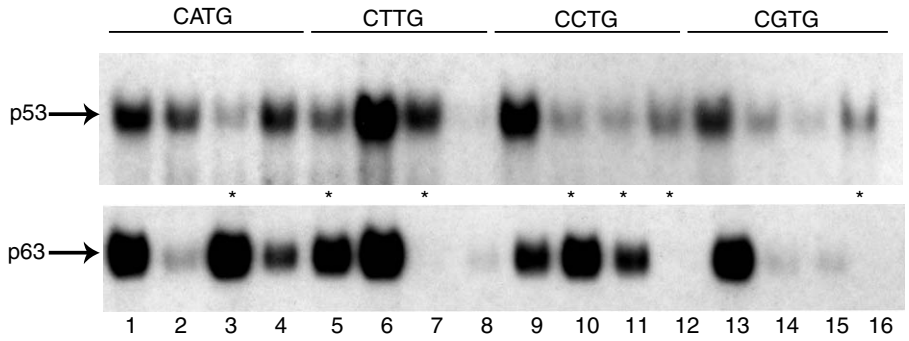


Fig. 4. Comparison of the DNA-binding specificity of p63 with p53 by EMSA. His-p63 and His-p53 proteins were each tested with the 16 oligonucleotides to compare the binding activities of the two proteins. Numbers on the bottom of each lane refer to the specific oligonucleotide used in this experiment. Only the DNA–protein complexes are shown. The asterisks indicate those lanes, which show significant difference in the binding activity between p63 and p53.

genes, there were many p53-like binding sites that were described, but these were not analyzed for p63 binding. Our new consensus could prove to be useful in detecting in silico the potential p63 binding sites that are present in regulatory regions for these genes as well as in future targets.

Similar to p53, the degenerate nature of the p63 DNA-binding sequence might be important in providing diversity and flexibility in transcriptional regulatory controls. However, this variability or degeneracy also makes it difficult to identify bona fide p63 binding sites in target genes. Our analysis suggests that although DNA-binding by p63 is flexible, there exists a distinct sequence signature that results in stronger binding specifically of p63. Importantly, this sequence signature consists of not only the core region but also flanking sequences surrounding the core. The availability of the p63 consensus sequence is a first step towards validating the in vivo targets of p63 and sorting out high affinity sites from those that are potentially weak.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.07.004.

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