Figure 2.4. **Mutations within the NH_2-terminal arm of AKR exhibit increased binding activities for wild- type and mutant binding sites.** EMSAs were carried out using GST fusions of the wild-type AKR or the NH_2-terminal mutant proteins along with a variety of binding sites containing mutations within the hexanucleotide core. A-D, both the G4R and R6K NH_2-terminal mutants were incubated in the presence of wt F', F'(G) and Opt-1 binding sites as well as a site where the G and A at positions 2 and 3 had been exchanged. E-G, binding of these mutant proteins was also examined in the presence of sites containing mutations at positions 1, 3 and 5 that abrogated binding by the wild-type protein. H, the NH_2-terminal mutants were also examined for alterations to their binding specificity by using a site with a hepatmer repeat of a typical homeodomain 5'-TAAT-3' core that is not recognized by the wild-type protein. The critical nature of the G residues at positions 2 and 9 to the binding of the two NH_2-terminal mutants was also examined. I, EMSA was carried out using a binding site where the G at position 9 was changed to T. J, a binding site was also used which contained an A at this position. K, alternatively binding of these mutants to a site containing a mutation of G2 to T was examined. L, finally, binding was examined to a site containing a mutation of both G2 and G9 residues, as found in the FM1 mutant site which was shown previously to be essential for binding of wild-type AKR.

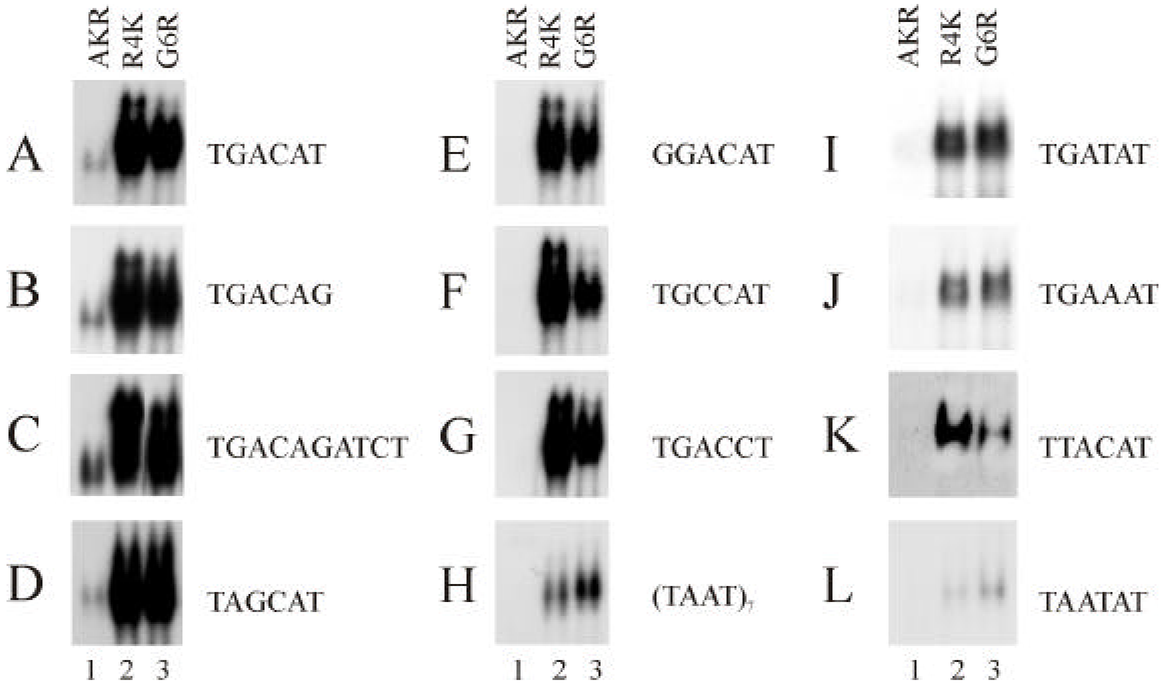
AKR
R4K
G6R
A
TGACAT
B
TGACAG
C
TGACAGATCT
D
TAGCAT
1
2
3
AKR
R4K
G6R
E
GGACAT
F
TGCCAT
G
TGACCT
H
(TAAT)$_7$
1
2
3
AKR
R4K
G6R
I
TGATAT
J
TGAAAT
K
TTACAT
L
TAATAT
1
2
3

Figure 2.5. **Helix 3 mutations validate the AKR/DNA complex model.** EMSAs were carried out using GST fusion proteins of either the wild-type or helix 3 mutants along with a variety of binding sites containing mutations within the hexanucleotide core. A-C, the N47I and I50K mutants were incubated with the wt F', F'(G) and Opt-1 binding sites. D, binding was examined using a site containing a G to T mutation at position 1. E and F, sites were also used which contained mutations at positions 1 and 2 or 3. G, the binding of these mutants was tested using a site that contained a G at position 2 or alternatively; H-I, sites containing G to A or G to T mutations at position 9. J, the R54M mutant was incapable of recognizing any of the sites tested, supporting the predicted importance of this residue for binding by the AKR homeodomain. Presented is a comparison of the binding of wild-type AKR and the R54M mutant to the wild-type F' site.

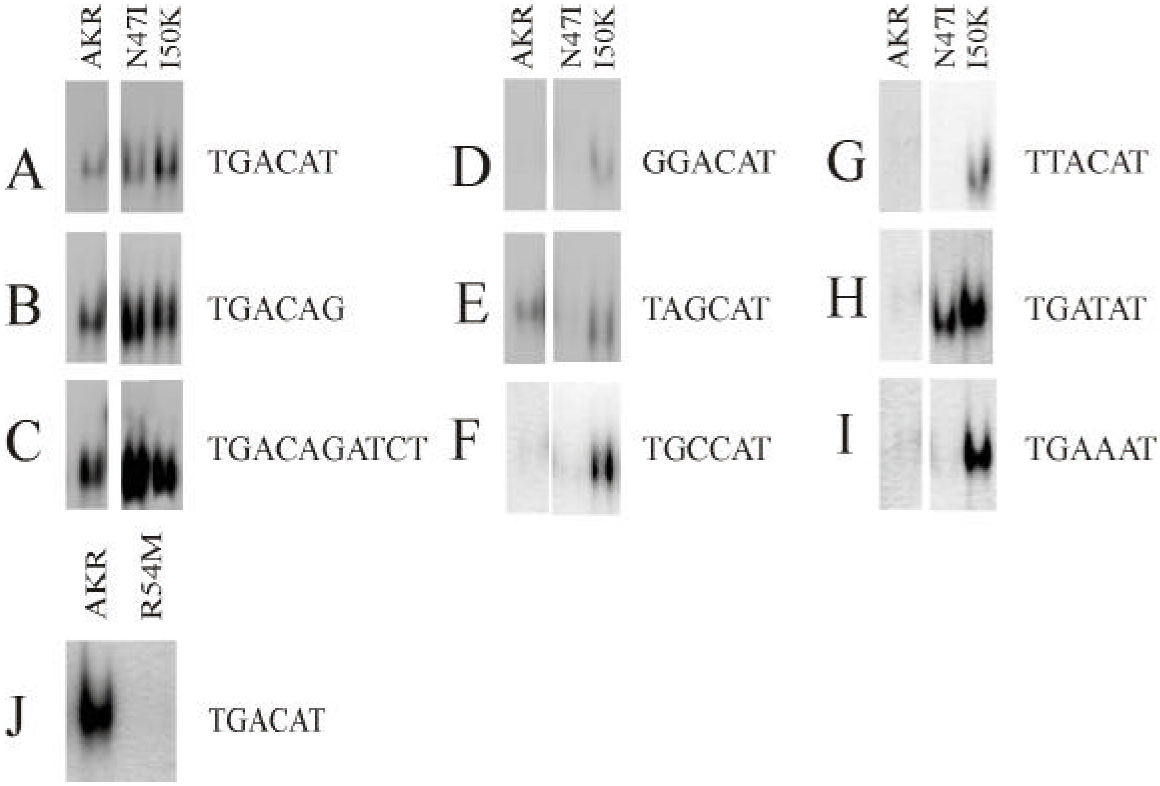
AKR
N47I
I50K
A
TGACAT
B
TGACAG
C
TGACAGATCT
AKR
N47I
I50K
D
GGACAT
E
TAGCAT
F
TGCCAT
AKR
N47I
I50K
G
TTACAT
H
TGATAT
I
TGAAAT
AKR
R54M
J
TGACAT

Figure 2.6. **The NH_2-terminal mutants suppress the estrogen-responsiveness of the F' and FM2 reporter constructs more actively than the wild-type factor.** A, luciferase reporter constructs were generated which had concatamers of the F', FM1, FM2 or Opt-1 binding site placed upstream of a minimal SV40 promoter. An alignment of the single binding site sequences are shown. B, These reporter constructs were transfected into the avian liver cell line, LMH/2A [337]. After a 24-hour incubation, the transfectants were treated with 1 ìM DES or an equivalent concentration of ethanol. Following a further 24-hour incubation, the cells were harvested and luciferase activity assayed (described under Materials and Methods). The luciferase reporter constructs (pGL2p, F', Opt-1) were also transiently co-transfected along with 0, 5 or 10 ng of; C, wild-type AKR; D, the R4K mutant or; E, the G6R mutant expression vectors. After a 24-hour incubation the transfectants were treated with 1 ìM DES and processed as described previously. These values were then expressed relative to the activities of the respective reporters in the absence of AKR and DES.

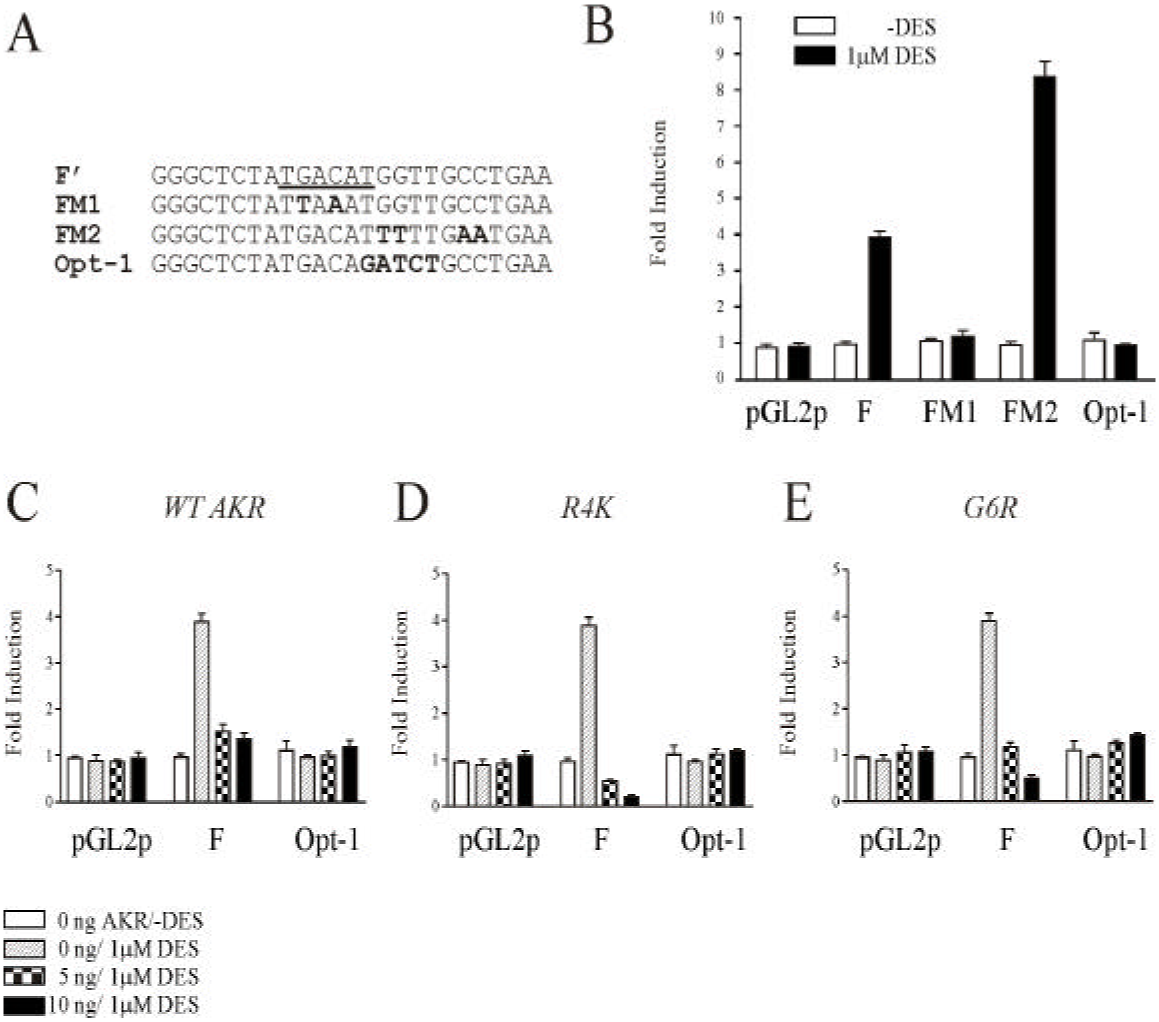
A
F′ GGGCTCTATGACATGGTTGCCTGAA
FM1 GGGCTCTATTAAATGGTTGCCTGAA
FM2 GGGCTCTATGACATTTTTGAATGAA
Opt-1 GGGCTCTATGACAGATCTGCCTGAA
B
-DES
1μM DES
Fold Induction
0
1
2
3
4
5
6
7
8
9
10
pGL2p
F
FM1
FM2
Opt-1
C
WT AKR
D
R4K
E
G6R
Fold Induction
0
1
2
3
4
5
pGL2p
F
Opt-1
0 ng AKR/-DES
0 ng/ 1μM DES
5 ng/ 1μM DES
10 ng/ 1μM DES

Table 2.1. Binding Site Oligonucleotides used in EMSAs presented in this study.

Binding Site	Function	Nucleotide Sequence[a,b]
WT F'	EMSA Kinetic Analyses	5'-AAAGGGGCCTCTATGACATGGTTGCCTGAA-3 3'-CGGAGATACTGTACCAACGGACTTTTACAT-5'
FM1	EMSA	5'-AAAGGGGCCTCTATTAAATGGTTGCCTGAA-3' 3'-CGGAGATAATTTACCAACGGACTTTTACAT-5'
FM2	EMSA	5'-AAAGGGGCCTCTATGACATTTTTGAATGAA-3= 3'-CGGAGATACTGTAAAAACTTACTTTTACAT-5'
Opt-1	EMSA	5'-GTTTATGAAAGGGGCCTCTATGACAGATCTGCCTGAAAATGTAGG-3' 3'-CAAATACTTTCCCCGGAGATACTGTCTAGACGGACTTTTACATCC-5'
WT F' Core	EMSA Kinetic Analyses	5'-GAAAGGGGCCTCTATGACATGGTTGCCTGAAAATGTAG-3' 3'-CCAACGGACTTTTACATC-5'
Core Mutant 1	EMSA	5'-GAAAGGGGCCTCTAGGACATGGTTGCCTGAAAATGTAG-3'
Core Mutant 2	EMSA	5'-GAAAGGGGCCTCTATGCCATGGTTGCCTGAAAATGTAG-3'
Core Mutant 3	EMSA	5'-GAAAGGGGCCTCTATGACCTGGTTGCCTGAAAATGTAG-3'
Core Mutant 4	EMSA Kinetic Analyses	5'-GAAAGGGGCCTCTATGACAGGGTTGCCTGAAAATGTAG-3'
Core Mutant 5	EMSA	5'-GAAAGGGGCCTCTATTACATGGTTGCCTGAAAATGTAG-3'
Core Mutant 6	EMSA	5'-GAAAGGGGCCTCTATGAAATGGTTGCCTGAAAATGTAG-3'
Core Mutant 7	EMSA	5'-GAAAGGGGCCTCTATGATATGGTTGCCTGAAAATGTAG-3'

[a] All oligonucleotides were synthesized using an Oligo 1000 DNA Synthesizer (Beckman).

[b] The regions representing the hexanucleotide core are underlined.

Table 2.2 Summary of Predicted Contacts derived from the AKR/Opt-1 Complex Model.

Residue	Atom	H-bond	Van der Waals
N47	(Oä1)	T8	T10 C5A, O4, C5 G9 C2', N9, N7
	(Nä2)	G9 O^2P	
I50	(Cγ)		G2 N7, C3 T1 C5, C6
	(Cδ)		G2 N7
N51	(Nä2)	G9 N7	G9 C5, N7, O6
	(Oä1)		T8 O4, C5A; G9 O6; C4 N4
	Câ		T8 C5A
	Cã		T8 C5A; G9 O6
R53	(Nç2)	G2 O^2P	T1 C2’, C3’
	(C2)		G2 O^2P
R54	(Nç1)		C4 N4; C4; C5
	(Nç2)	T8 O4; A5 N6; C4 N4	C4 C4; G9 O6; A5 C6
	(Cî)		T8 O4; A5 N6; C4 C4, N4
	(Nå)		C4 N4
R55	(Nç1)	C7 O5’	C7 C5’
	(Nç2)	C7 O5’	C7 C5’
	(Cî)	C7 O5’	C7 C5’
I47	Câ		T10 C5A
	Cã1		T10 C5A
	Cã2		T10 C5A, O4
	Cä		T10 C5A; G9 C2', O^2P; C8
K50	(Nî)	G2 O6	T1 04, C4, C5; G2 N7, C6
	Cå		G2 O6 N7
	Cä		T1 C5, C6; G2 N7
K54			C4 C5
M54			C4 N4

In order to facilitate reading of this table, an example is provided. The Oä1 of Asn47 forms a hydrogen bond with T8 of Opt-1. Oä1 also establishes van der waals interactions with C5A, O4 and C5 at distances of T10 as well as C2’, N9 and N7 of G9. Lastly, Nä2 of Asn47 establishes a hydrogen bond to G9.

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