6.2. Bandshift-assays

6.2.1. Bandshift assays with different oligonucleotides

The range of oligonucleotides bound by Zα-peptide was investigated by performing bandshift assays with different oligodeoxynucleotide substrates. The results are summarized in Table 6.1 . Most of the oligodeoxynucleotides used were so-called hairpin molecules in which the two complementary strands of double stranded DNA are connected by a loop of four thymidine deoxynucleotides. Both linear and hairpin molecules containing CG or GC dinucleotide motifs were bound with the exception of the hairpin molecules $(GC)_3ACGCT_4GCGT(GC)_3$ and $(GC)_3(CG)_3T_4(CG)_3(G)_3$. Hairpins made up of GT/AC or AT dinucleotide repeats were not bound by GST-Zα.

DNA Substrates bound by GST-Zα	DNA Substrates not bound by GST-Zα
linear molecules : $((CG)_8)_2$ $((GC)_8)_2$	
hairpin molecules: $(CG)_3T_4(CG)_3$ $(CG)_4T_4(CG)_5$ $(CG)_5T_4(CG)_5$ $(CG)_6T_4(CG)_6$ $(CG)_8T_4(CG)_8$ $(CG)_9T_4(CG)_9$ $(CG)_{12}T_4(CG)_{12}$ $(GC)_4T_4(GC)_4$ $(GC)_5T_4(GC)_5$ $(GC)_6T_4(GC)_6$ $(GC)_8T_4(GC)_8$ $T(GC)_4T_4(GC)_4$ $(GC)_4T_4(GC)_4TT$ $AC(GC)_3T_4(GC)_3GT$	$(GC)_3ACGCT_4GCGT(GC)_3$ $(GC)_3(CG)_3T_4(CG)_3(GC)_3$ $(GT)_6T_4(AC)_6$ $(AT)_6T_4(AT)_6$

Table 6.1 Summary of the bandhift assays. Oligodeoxynucleotides of different sequences were chemically synthesized, radioactively end labeled and used in bandshift assays with between 0.5 and 1.0 µg GST-Zα peptide. The DNA:GST-Zα peptide complexes were resolved on 5 % (w/v) polyacrylamide gels and visualized by autoradiography.

6.2.2. Competition experiments with DNA and RNA

In order to more closely characterize the interaction between GST-Zα peptide and DNA a competition experiment was done in which a radioactively labeled hairpin $(CG)_4T_4(CG)_4$ was competed with different amounts of poly(CG)·poly(CG) and poly(m^5CG) · poly(m^5CG) (Figure 6.4). Even low amounts of both polymers led to a complete loss of binding visible as the lack of a shifted band. Therefore, stabilization of the DNA probe in the Z-DNA conformation by chemical modification of the DNA is not necessary in order to obtain a band shift with the GST-Zα peptide.

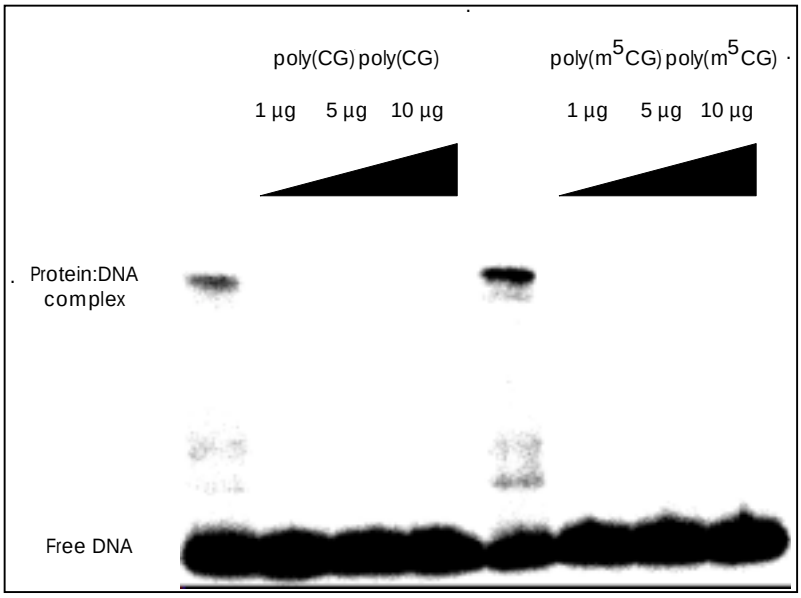

Figure 6.4 Competition of binding of the Zα peptide to $(CG)_4T_4(CG)_4$ with poly(CG) polymers. Radioactively end labeled hairpin $(CG)_4T_4(CG)_4$ was incubated with 500 ng Zα peptide. Different amounts of poly(CG)·poly(CG) and poly(m^5CG) · poly(m^5CG) were added as competitor. The DNA:GST-Zα peptide complexes were resolved on a 5 % (w/v) polyacrylamide gel and visualized by autoradiography. The positions of the Protein:DNA complex and the free DNA are indicated next to the autoradiograph. The amount of competitor DNA is indicated above each lane.

6.2.3. Competition with plasmid DNA

In order to show that binding of GST-Zα peptide to DNA is dependent on the Z-DNA conformation, binding to the $(CG)_4T_4(CG)_4$ hairpin was competed with three different plasmids whose only difference was the absence (pME.A) or presence (pME.C and pME.E) of sequences likely to adopt the Z-DNA conformation under the amount of negative supercoiling present in bacterial plasmids. Figure 6.5 shows a bandshift assay, in which a radioactively labeled hairpin $(CG)_4T_4(CG)_4$ was incubated with 0.7 µg GST-Zα. Different amounts of different plasmids were added as competitor DNA. While 1 µg of plasmids pME.C and pME.E led to a complete loss of binding, addition of 1 µg of plasmid pME.A resulted in only a partial loss of binding.

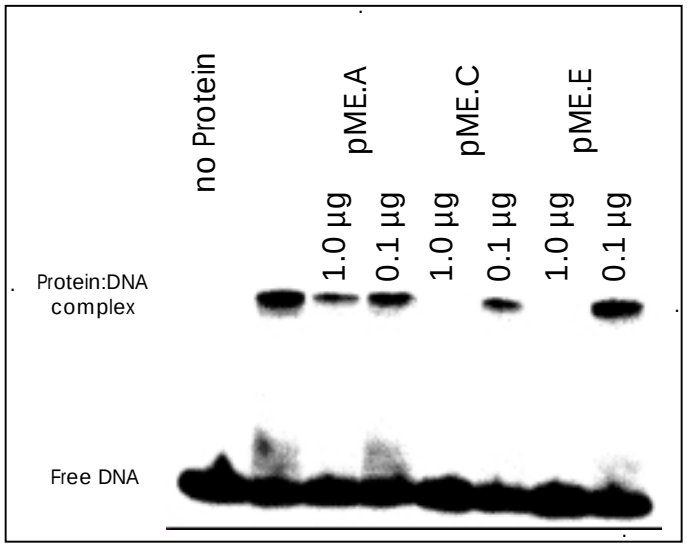

Figure 6.5 Competition of binding of the Zα peptide to $(CG)_4T_4(CG)_4$ with plasmid DNA. Radioactively end labeled hairpin $(CG)_4T_4(CG)_4$ was incubated with 500 ng Zα peptide. Different amounts of the plasmids pME.A, pME.C and pME.E were added as competitor. The DNA:GST-Zα peptide complexes were resolved on a 5 % (w/v) acrylamide gel and visualized by autoradiography. The positions of the Protein:DNA complex and the free DNA are indicated next to the autoradiograph. The type and amount of competitor DNA is indicated above each lane.

6.2.4. Competition with RNA

In order to investigate whether the Zα domain of ADAR1 can bind to RNA, a competition experiment was performed using poly r(A) and tRNA as competitor RNA´s. As before, 0.7 µg of His-Zα peptide were incubated with radioactively labeled hairpin $(CG)_4T_4(CG)_4$. Different amounts of poly(rA) or tRNA were added as competitor. Whereas addition of poly(rA) had no effect on the binding of the hairpin , addition of a large excess of tRNA lead to a loss of the shifted band.

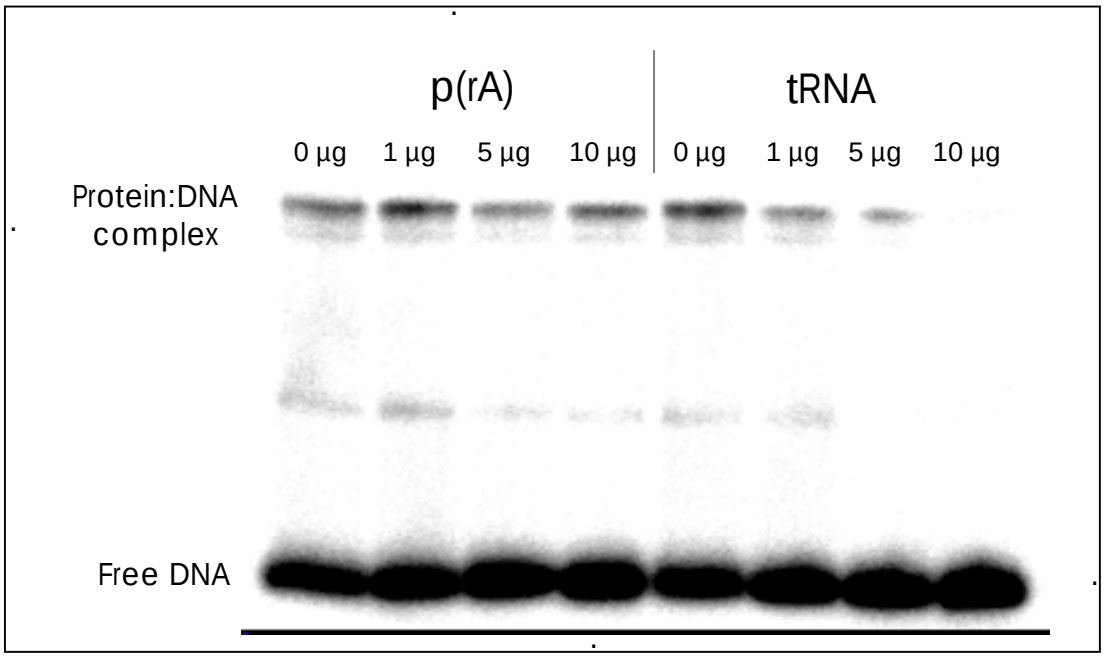

Figure 6.6 Competition of binding of the Zα peptide to $(CG)_4T_4(CG)_4$ with RNA. Radioactively end labeled hairpin $(CG)_4T_4(CG)_4$ was incubated with 500 ng Zα peptide. Different amounts of p(rA) and tRNA were added as competitor. The DNA:GST-Zα peptide complexes were resolved on a 5 % (w/v) polyacrylamide gel and visualized by autoradiography. The positions of the Protein:DNA complex and the free DNA are indicated next to the autoradiograph. The type and amount of competitor DNA is indicated above each lane.

6.2.5. Methylated DNA

Bandshift assays were done using DNA oligonucleotides methylated at the C5 position of all cytosine bases. For comparison, non-methylated oligonucleotides of identical sequence were included. Both a dodecamer $(m^5CG)_6$ and a hairpin $(m^5CG)_3T_4(m^5CG)_3$ gave rise to a bandshift upon addition of 500 ng Zα-peptide (Figure 6.7). The shifted band obtained with the dodecamer was more intense than that obtained with the hairpin oligonucleotides. Likewise methylation led to more DNA being shifted. Using the dodecamer several shifted bands were obtained, indicating that more than one Zα-peptide molecule can bind to this DNA oligomer.

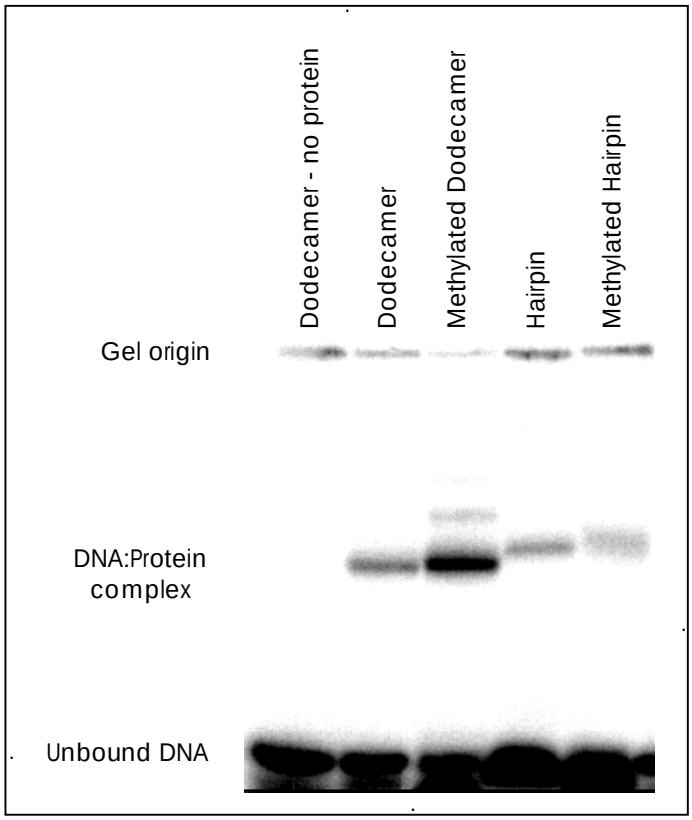

Figure 6.7 Binding of the Zα peptide to methylated DNA. Different methylated and non-methylated DNA molecules were radioactively end labeled and incubated with 0.5 µg His-Zα peptide. The DNA:His-Zα peptide complexes were resolved on a 5 % (w/v) acrylamide gel and visualized by autoradiography. The positions of the Protein:DNA complex and the free DNA are indicated next to the autoradiograph. The type of the DNA is indicated above each lane.

6.2.6. Mutations of the Hairpin sequence

6.2.6.1. Phased AT mutations

A series of DNA oligonucleotides whose sequence was derived from the hairpin sequence $(CG)_4T_4(CG)_4$ were used in bandshift assays Each oligonucleotide contained a single change as compared to this sequence. Positions 2 to 7 were mutated in such a way, that guanosine was replaced by adenosine and cytosine replaced by thymidine. The base in the opposing strand of the stem was replaced likewise, thereby retaining the base pairing of the stem. Oligonucleotides were end-labeled with [γ-^{32}P]-ATP and purified. The specific activity of the labeled oligonucleotides was adjusted with unlabeled oligonucleotide. Samples were incubated with 500 ng Zα-peptide and resolved on a 4 % (w/v) native polyacrylamide gel (Figure 6.8).

An intense shifted band was observed for those mutants that contain mutations at positions 2 or 7. Mutations at position 5 and 6 resulted in a shifted band of much weaker intensity (lanes 5 and 6). No shifted band was observed for those sequences that were mutated at positions 2 and 3 (lanes 2 and 3). In summary, it appears that mutations near the end of the sequences are tolerated better than those in the middle of the sequences.

6.2.6.2. Phased GC mutations

Another set of oligonucleotides derived from the $(CG)_4T_4(CG)_4$ hairpin contained mutations that interrupted the alternation of purine and pyrimidine bases. The sequences were changed such that at positions 2 to 6 guanosine was replaced by cytosine or cytosine replaced by guanosine. As in the case of the AT mutations, the opposing base of the stem was changed likewise in order to maintain base pairing. The oligonucleotides were radioactively end-labeled and the specific activity adjusted.

Whereas the un-mutated sequence led to a strong shifted band, destruction of the alternation of purine and pyrimidine bases had a significant effect on the intensity of the shifted band. A mutation at position 2 reduced the intensity of the shifted band considerably (lane 7). Mutations at position 4 or 6 lead to a weak signal (lanes 9 and 11) and mutations at positions 3 or 5 lead to almost complete loss of the shifted band (lanes 8 and 10). As was observed with the phased AT mutations, those mutations positioned near the ends of the sequence are better tolerated than those in the middle of the sequence.

However, the middlemost position is an exception, since a mutation at this position still allowed for binding.

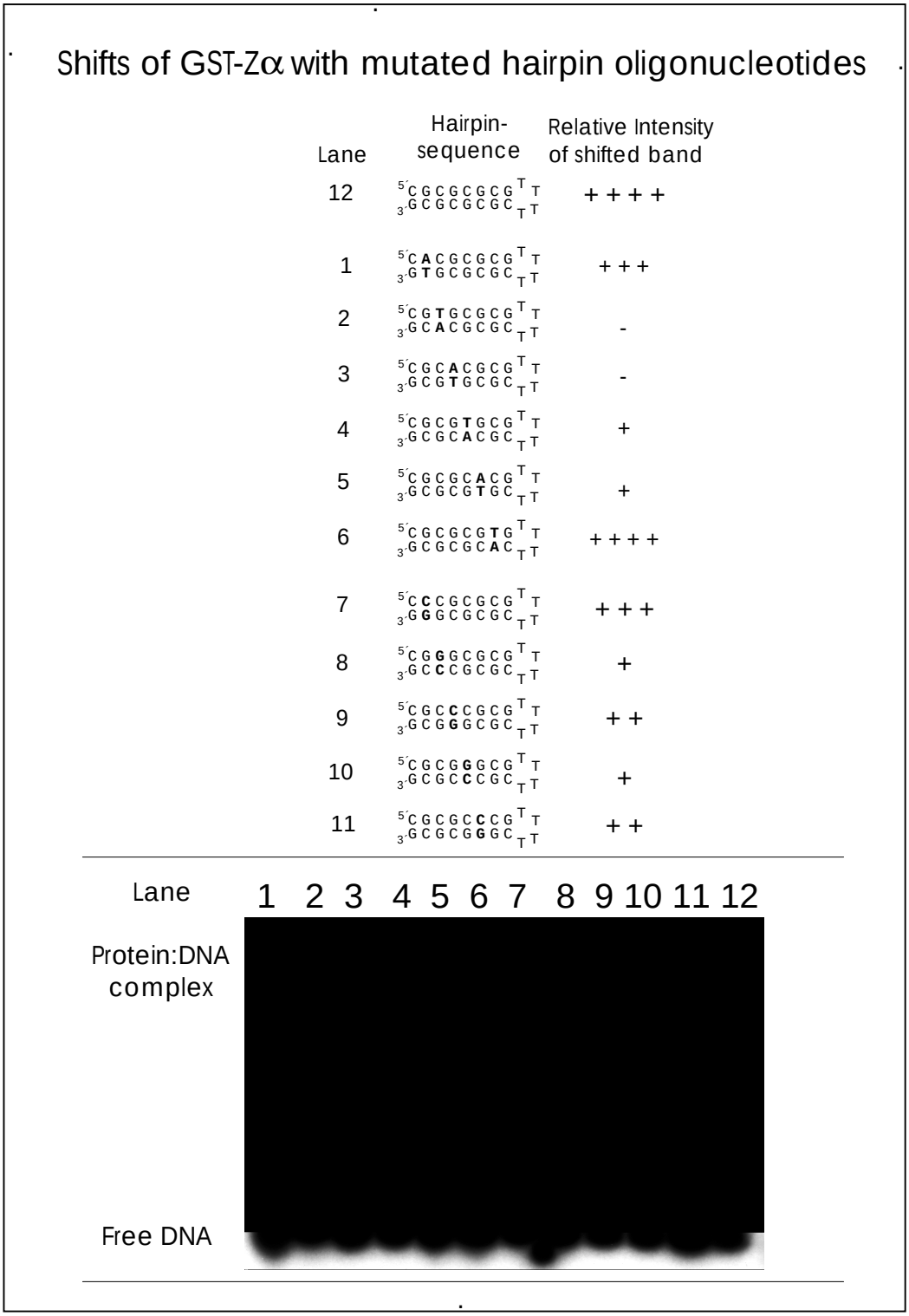

Figure 6.8. Bandshift assays with hairpin oligodeoxynucleotides derived from the $(CG)_4T_4(CG)_4$ sequence. Hairpin oligodeoxynucleotides with different sequences were radioactively end labeled and incubated with 0.5 µg GST-Zα peptide. The DNA:GST-Zα peptide complexes were resolved on a 5 % (w/v) acrylamide gel and visualized by autoradiography. The positions of the Protein:DNA complex and the free DNA are indicated next to the autoradiograph. The sequences of the hairpins are indicated for each lane in the table above the autoradiograph. The mutated positions are indicated with bold letters. A classification of the observed intensity of the shifted band is included as well.