Abstract

DNA synthesis is a complex process that is regulated at the stage of initiation of DNA replication. In bacteria, replication initiation occurs at the origin of replication (*ori*), where multiple molecular interactions occur. Our model system to study replication initiation is the *Escherichia coli* plasmid, R6K. The initiation of R6K replication occurs at γ *ori*, where there is an A+T rich region followed by seven binding sites for the replication initiator protein, π. π has two functions: monomers of the protein activate γ *ori* while dimers inhibit replication. We hypothesize that variations in the DNA sequences of the seven π-binding sites (iterons) at γ *ori* and an eighth iteron downstream of γ *ori* are important for π monomer and/or dimer binding. To test this hypothesis, the affinity of each of the different iterons for π was studied both *in vivo* and *in vitro*. The *in vivo* assay monitored the relative affinity of each iteron for π in competition with the full γ *ori*. In the *in vitro* assay, electrophoretic mobility shift assay was used to quantify π binding affinity with or without a competitor iteron. Results suggested that π binds to different iterons with different affinities, with strongest binding to the iteron having the most consensus sequence, and weakest binding to the iteron with the least consensus sequence.

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COVER SHEET

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Protein-DNA Interactions Involved in the Initiation of DNA Replication

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Abstract

DNA synthesis is a complex process that is regulated at the stage of initiation of DNA replication. In bacteria, replication initiation occurs at the origin of replication (*ori*), where multiple molecular interactions occur. Our model system to study replication initiation is the *Escherichia coli* plasmid, R6K. The initiation of R6K replication occurs at γ *ori*, where there is an A+T rich region followed by seven binding sites for the replication initiator protein, π. π has two functions: monomers of the protein activate γ *ori* while dimers inhibit replication. We hypothesize that variations in the DNA sequences of the seven π-binding sites (iterons) at γ *ori* and an eighth iteron downstream of γ *ori* are important for π monomer and/or dimer binding. To test this hypothesis, the affinity of each of the different iterons for π was studied both *in vivo* and *in vitro*. The *in vivo* assay monitored the relative affinity of each iteron for π in competition with the full γ *ori*. In the *in vitro* assay, electrophoretic mobility shift assay was used to quantify π binding affinity with or without a competitor iteron. Results suggested that π binds to different iterons with different affinities, with strongest binding to the iteron having the most consensus sequence, and weakest binding to the iteron with the least consensus sequence.

Introduction

Replication of DNA is a complex and highly regulated process that is a prerequisite for cell division in all species. Studying the mechanisms involved in the initiation of DNA replication will expand the understanding of the nature of protein-protein and protein-DNA interactions. Furthermore, understanding bacterial DNA replication may lead to new targets for anti-microbial agents.

We study replication of the *Escherichia coli* plasmid, R6K. Like other plasmids, R6K is a self-replicating, extra-chromosomal piece of DNA. R6K is maintained at a copy number of approximately 15 per cell. Two plasmid-encoded elements are necessary for this precise control of replication: the γ origin of replication (γ *ori*) and the replication initiator protein, π. During the initiation of R6K replication, π binds to γ *ori* to facilitate the opening of the double-stranded DNA. Within γ *ori*, there are seven 22-base-pair (bp) reiterated DNA sequences called "iterons", which act as binding sites for π. The iterons are similar to each other but not identical. The second and the fifth iterons have the most consensus sequence while iteron number four has the least consensus sequence. Further from the seven-iteron region, there is an eighth iteron whose function in the initiation of replication is not certain. The location of the eight iterons and the variations in their DNA sequences are shown in Figure 1.

π protein exists in both the monomeric and dimeric forms, and although wild-type π is predominantly dimeric in solution, both forms of π can bind to the iterons. However, only monomers activate γ *ori* while dimers inhibit replication.[1-5] We hypothesize that variations in the DNA sequences of the eight iterons at γ *ori* are important for π monomer

and/or dimer binding. To test this hypothesis, the affinity of π for each of the six different iterons was studied both *in vivo* and *in vitro*.

Materials and Methods

Bacterial Growth

E. coli strain ECF001containing plasmid pFW25 and derivatives of plasmid pUC9 was grown at 37°C in Luria-Bertani (LB) liquid medium with the addition of penicillin (PEN) at 750 μg/mL, chloramphenicol (CAM) at 15 μg/mL, and arabinose at 0.02%. The same strain was grown at 37°C on LB agar with PEN at 750 μg/mL and CAM at 15 μg/mL.

Oligos and Plasmids

Each of the different iterons from γ *ori* (iterons number one, two, three, four, seven, and eight) were cloned into both pUC9 and pBend5 plasmids (iterons number five and two are identical, and iterons number six and one are identical). To do this, six pairs of oligos were purchased from IDT (Coralville, IA). Each pair consisted of the top and bottom strand of one iteron (their sequences are shown in Figure 1). The top and bottom strand of each pair were annealed and the double-stranded DNA was ligated into the *Hinc*II site of pUC9 (Promega, Madison, WI) following standard cloning procedures[6] for the *in vivo* assay. The plasmids were extracted using a Miniprep kit (QIAGEN, Valencia. CA). pBend5 derivatives for the *in vitro* assay were prepared by ligating each double-stranded iteron into the *Hpa*I site of pBend5.[7] Standard cloning procedures were followed and the plasmids were extracted using a Midiprep kit (QIAGEN, Valencia, CA).

In vivo π/Iteron Binding Assay

A plasmid containing γ *ori* (pFW25), was established in the *E. coli* host strain ECF001 (Figure 2). 75 ng of the pUC9 derivative, which contained one of the six different iterons, was introduced into the competent host cells by standard transformation. LB broth was then added to a final volume of 1 mL followed by a 45-minute incubation at 37°C. From each test tube, a 1:20 dilution was made and 100 μL from each dilution was spread onto a LB agar plate containing PEN, as well as a LB agar plate containing PEN and CAM. The plates were incubated at 37°C for 16 to 20 hours, and the number of colonies on each plate was counted.

Preparation of Radioactive Fragments Containing One Iteron

pBend5 derivative plasmids were cut with *EcoR*V and the resulting 149-bp fragments with one iteron were purified by a gel extraction kit (QIAGEN, Valencia, CA) from a 6% polyacrylamide gel. The six different fragments were then end-labeled with [γ^{32}P]ATP and purified by using a G50 column (Amersham Biosciences, Piscataway, NJ).

Quantification of DNA

***In vitro* π/Iteron Binding Assay**

80 pg of each labeled iteron was mixed with 65 ng of polydIdC and binding buffer (2 mM Tris-HCl (pH 7.5), 0.6 mM $MgCl_2$, 0.1 mM EDTA, 10 mM potassium glutamate) to a final volume of 14 μL. It was followed by adding 1 μL of protein (prepared as previously described)[8,9] in TGE buffer (10 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, and 0.3 M KCl) with increasing concentration from 0 ng to 350 ng. The mixture was incubated at room temperature (27°C) for 15 minutes. After adding 3 μL of loading

dye, the samples were loaded onto a 6% polyacrylamide gel which had been pre-electrophoresed at 150 V for one hour. The samples were run at 180 V for one hour. The gels were vacuum-dried for 30 minutes, exposed to a phosphor screen for 12 to 16 hours, and observed under a Typhoon™ phosphorimager (GE Healthcare). Finally, the radioactive bands were quantified with the computer software Image Quant (Molecular Dynamics).

In vitro Competition Assay

The *in vitro* competition assay differed from the above *in vitro* π/iteron binding assay as follows: 40 pg of labeled iteron number two was used instead of 80 pg of labeled iterons, 32.5 ng of polydIdC was added instead of 65 ng, and the 14-μL mixture contained 1 μL of increasing amount (from 0 ng to 75ng) of pBend5 derivative plasmids. Finally, 250 ng of protein was added to each reaction tube instead of a varying amount. In this assay, the gels were exposed to a phosphor screen for three to four days.

Binding Affinity Calculation

The computer software KaleidaGraph (Reading, PA) was used to determine the π binding affinity for different iterons. Data from the *in vitro* assay were fitted to the equation of Langmuir isotherm:[10]

$$¥ = k_1 L / (1 + k_1 L)$$

where ¥ is the fraction of bound DNA, k_1 is the binding affinity constant, and L is the protein concentration.

Results

In vivo π/Iteron Binding Assay

The *in vivo* π/iteron binding assay (Figure 2) was performed in order to compare the affinity of each iteron for π in the presence of other cellular factors. In this assay, pFW25, a γ *ori* plasmid, that requires π to replicate, was established in a specially-engineered *E. coli* strain that produces π chromosomally. This plasmid has a CAM resistance marker. Another plasmid, containing one of the six different iterons, was introduced into the cells by standard calcium chloride transformation. This PEN-resistant plasmid contains the pUC *ori*, so it does not require π for replication. The strength of π binding to the one-iteron-containing pUC9 derivative would determine if there was enough π left for the replication of pFW25. In theory, a stronger iteron in the pUC9 derivative would titrate more π and, as a result, the two plasmids would be less likely to co-exist in the cell. Consequently, fewer colonies would arise on the agar plate with both antibiotics. A weaker iteron in the pUC9 derivative would leave more π for pFW25, and the two plasmids would be more likely to co-exist in the cell. Consequently, more colonies would be found on the agar plate containing both antibiotics. Therefore, the number of colonies would be inversely related to the affinity of π for the iteron being tested.

The results showing the average number of colonies on each plate are presented in Table 1. The LB agar plate containing PEN (PEN Only) was a standard showing the transformation efficiency for each case, while the LB agar plate containing PEN and CAM (PEN + CAM) plate indicated cells having both plasmids. The final percentage was

a ratio of the number of colonies that arose on the PEN + CAM plate to those of the PEN only plate. The percentages were compared for different incoming pUC9 derivatives. While iteron numbers one, two, three, four and seven gave rise to similar percentages, iteron number eight resulted in a much lower percentage (19.3%) of colonies.

In vitro π/Iteron Binding Assay

The *in vitro* π/iteron binding assay was performed to eliminate unknown cellular factors and focus only on π/iteron interactions. Electrophoretic mobility shift assay (EMSA) was used to study the binding affinity of each different iteron for π. EMSA is the separation of particles of different molecular weights based on their relative mobility through a polyacrylamide gel. Thus, π-DNA complexes were separated from free DNA. Figure 3 shows the EMSA results for each of the six different iterons. The free DNA, monomer-bound DNA and dimer-bound DNA bands were labeled. The darkness of the bands corresponded to the relative amount of DNA present. The calculated binding affinity of each iteron for π is shown in Figure 4. Since the iterons' affinity for monomers and dimers of π could not be computed separately, the overall affinity of each iteron for π was determined. The values represent the relative affinity of each iteron for π: the larger the number, the higher the affinity. It is seen that iterons number two and eight have relatively high values, while iteron number four has a relatively low value.

In vitro Competition Assay

To study the interactions of π with iterons on plasmid DNA as compared to linear DNA, an *in vitro* competition assay was performed. In this assay, derivatives of the

plasmid pBend5 containing individual iterons were introduced to the reaction mixtures to compete for π with the radioactively-labeled fragment containing iteron number two. A stronger iteron on pBend5 would titrate more π and, as a result, there would be less π to bind the radioactive linear DNA. Consequently, the bands representing π-DNA complexes would decrease in intensity. On the other hand, a weaker iteron on pBend5 would leave more π to bind the radioactive linear DNA, resulting in stronger bound-DNA bands.

The results of this experiment are shown in Figure 5. The percentage of monomer-bound DNA generally decreases with increasing competitor DNA, while the percentage of dimer-bound DNA generally increases with increasing competitor DNA. The overall percentage change of monomer-bound DNA and dimer-bound DNA was calculated for each iteron. The greater the percentage decrease in monomer-bound DNA, the stronger the iteron is as a binding site for π monomers. Iterons number two and eight appear to be the strongest π binding sites, while iteron number four is the weakest.

Discussion

There are variations in the sequences of the eight iterons at γ *ori*, and the results suggest that the different iterons do not have the same affinity for π. Specifically, from the *in vivo* results, the number of colonies with pUC9 containing iteron number eight is relatively lower, suggesting that iteron number eight is one of the strongest binding sites for π. However, the cell is a complicated system and includes many factors that can affect π binding, thus the *in vivo* results may or may not be directly related to the actual affinity of the different iterons for π. Results from the *in vitro* π/iteron binding assay suggest that the two iterons with the strongest affinities for π are iterons number eight and two respectively, while the weakest binding occurs at iteron number four. Moreover, preliminary results from the *in vitro* competition assay also suggest that π binds differently to different iterons. In fact, the plasmids containing iterons number two and eight were the best competitors, meaning that π possibly strongly binds to these two iterons. The plasmid that competed for π the least contained iteron number four, suggesting that π binds weakly to that iteron.

In conclusion, the variations in DNA sequence among the iterons play a role in π affinity. Iteron number two, which has the most consensus sequence, appears to have the strongest affinity for π. On the other hand, iteron number four, having the most degenerate sequence, appears to have the weakest affinity for π. The different affinity of the eight iterons for π in the γ *ori* may be important in opening the double-stranded DNA. Further experiments should be carried out in order to obtain more statistically significant results.

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A pUC 9 derivative	B PEN Only	C PEN + CAM	D Ratio (C/B)
Iteron #1	442	142	32.1%
Iteron #2	442	112	25.3%
Iteron #3	509	181	35.6%
Iteron #4	346	85	24.6%
Iteron #7	552	142	25.7%
Iteron #8	337	65	19.3%

Table 1. Results from the *in vivo* π/iteron binding assay. Column (A) shows the iteron contained in the incoming pUC9-derivative plasmid. Columns (B) and (C) show the average number of colonies (three trials) that arose on PEN Only plates and PEN + CAM plates, respectively. Column (D) shows the ratio of column C to column B in percentages. In general, iteron number eight gave rise to a smaller number of colonies.

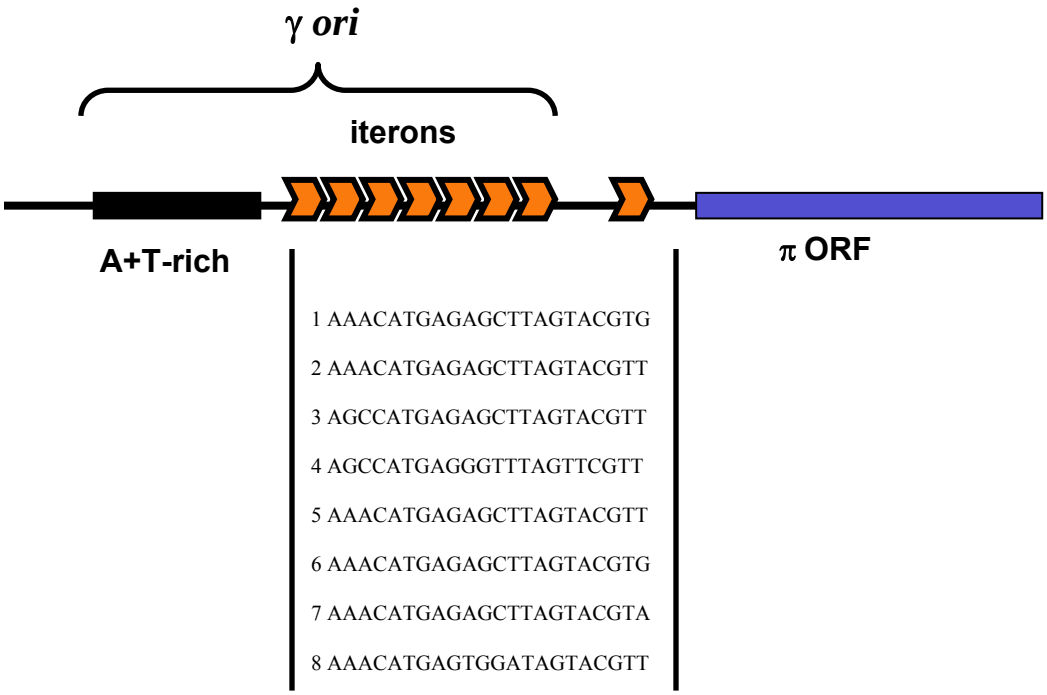

Figure 1. γ *ori* in R6K. The figure shows the location of the seven iterons in γ *ori*, as well as an eighth iteron downstream of γ *ori*. They are represented by orange arrows and the top strands of their sequences are shown under the arrows. ORF stands for open reading frame.

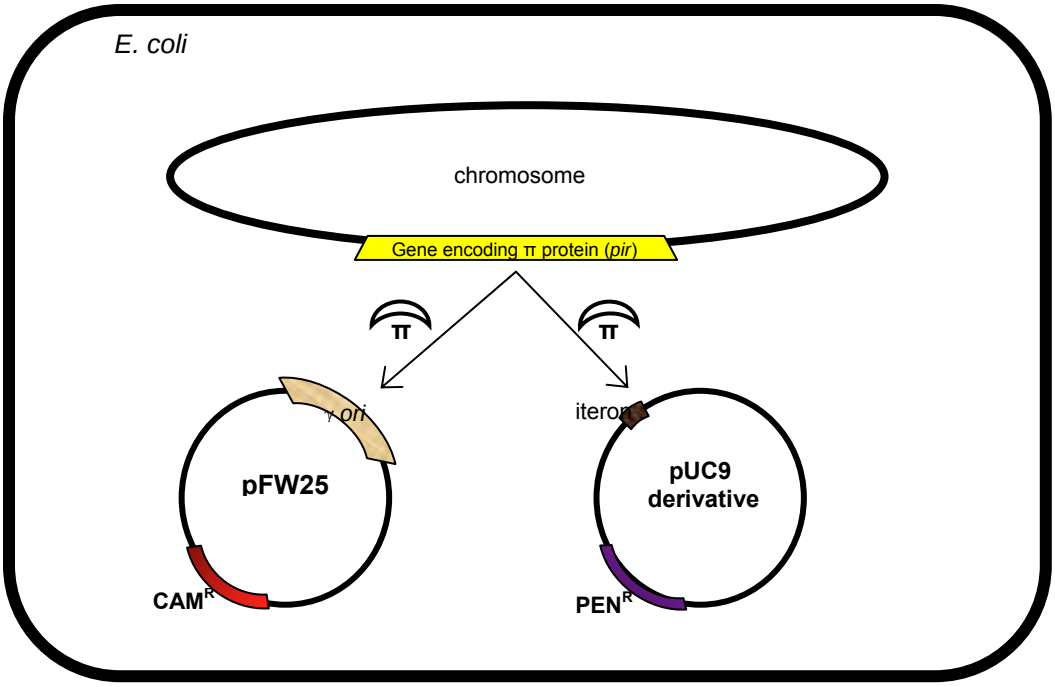

Figure 2. The *in vivo* π/iteron binding assay. The two plasmids present in the cell have different antibiotic resistance markers. π is required for the replication of the γ *ori* plasmid but unnecessary for the incoming plasmid. The stronger the binding site on the incoming plasmid, the more π it will titrate, and the less likely the two plasmids will co-exist. Therefore, fewer colonies will arise on the agar plate with both antibiotics.

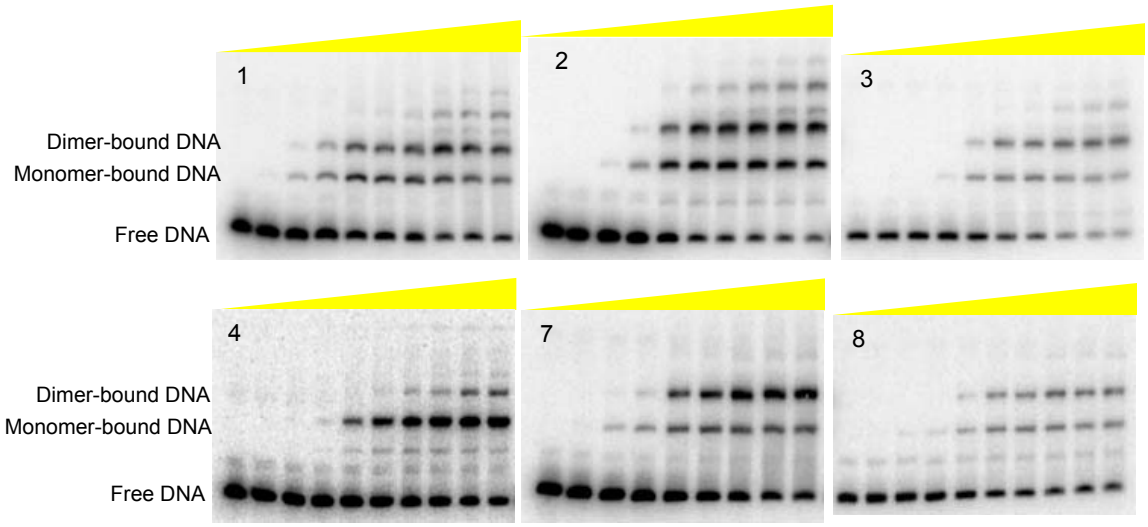

Figure 3. EMSA results from the *in vitro* π/iteron binding assay. Numbers on the left-hand corner of each gel represent the iteron number tested. The free DNA, monomer-bound DNA and dimer-bound DNA bands are labeled. The triangle above each gel represents the increasing concentration of π. The specific concentrations (in ng/μL) are as follows (from left to right): 0, 12.5, 25, 50, 100, 150, 200, 250, 300, 350. Iteron number seven did not have the 350-ng/μL lane.

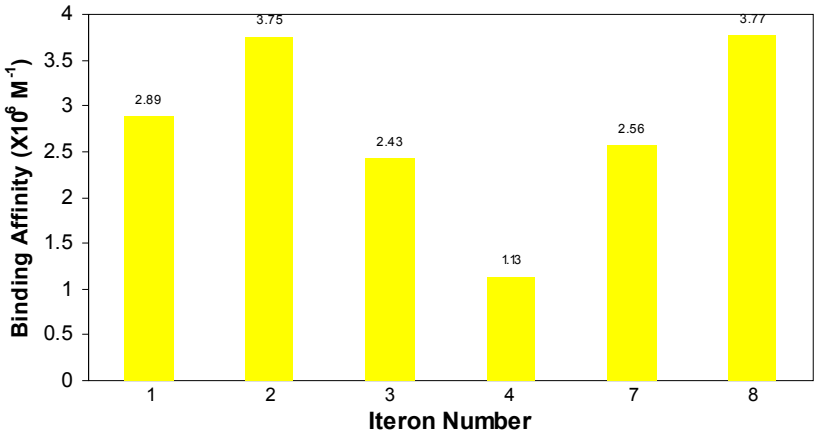

Figure 4. π binding affinity for each of the different iterons. The bands in Figure 3 were quantified and the fraction of bound DNA (monomers + dimers) was plotted on a graph and subjected to a best-fit curve using the Langmuir isotherm (See Materials and Methods) to reveal the binding affinity for each iteron. Higher values correspond to higher affinity of π. The values were based on the average of three trials for iterons number one, three seven and eight, two trials for iteron number four, and one trial for iteron number two.

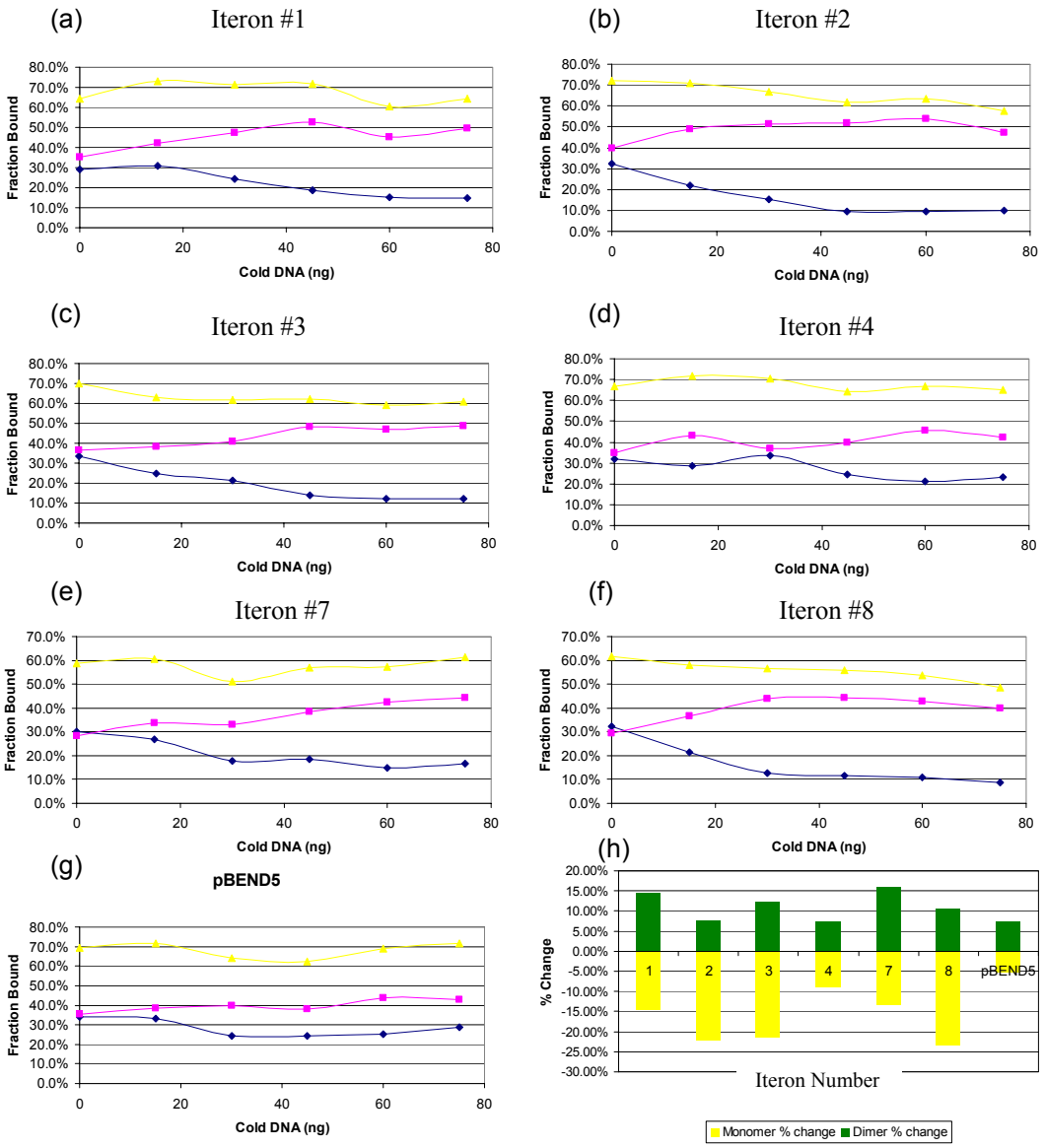

Figure 5. Results from the *in vitro* competition assay. Panels (a) through (g) show the percentage change of monomer- and dimer-bound linear DNA with increasing competitor plasmid DNA for each of the different iterons (or no iteron as in (g)). The data was obtained from one representative trial of the EMSA results. The blue diamond line represents the percentage of monomer-bound linear DNA, the purple square line represents the percentage of dimer-bound linear DNA, and the yellow line is the total bound DNA. Panels (a), (b), (c), (d), and (g) have graphs with y-axis from 0% to 80%, while panels (e) and (f) have graphs with y-axis from 0% to 70%. Panel (h) shows the overall percentage change of monomer- and dimer-bound DNA for each iteron, represented by the yellow and green bars, respectively.