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Molecular flip–flops formed by overlapping Fis sites

Paul N. Hengen, Ilya G. Lyakhov, Lisa E. Stewart[1] and Thomas D. Schneider[1,*]

Intramural Research Support Program, SAIC and [1]Laboratory of Experimental and Computational Biology, NCI Frederick, Frederick, MD, USA

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ABSTRACT

The DNA-binding protein Fis frequently uses pairs of sites 7 or 11 base pairs (bp) apart. Two overlapping Fis sites separated by 11 bp are found in the *Escherichia coli* origin of chromosomal replication. Only one of these sites is bound by Fis at a time, so the structure is a molecular flip–flop that could direct alternative firing of replication complexes in opposite directions. Alternatively, the flip–flop could represent part of an on–off switch for replication. Because they can be used to create precise switched states, molecular flip–flops could be used as the basis of a novel molecular computer.

INTRODUCTION

Fis is a well characterized site-specific DNA-binding protein which is known to bend DNA and is involved in many site-specific recombination systems (1). In addition, it autoregulates its own promoter and activates other promoters (2,3). When *Escherichia coli* encounters a rich nutritional medium, the number of Fis molecules increases from nearly zero to 25 000–50 000 dimers per cell (4). Estimates of the number of Fis sites in the *E.coli* genome based on the average information in Fis sites give a similar number, indicating that most Fis molecules are controlling genetic systems throughout the genome (5–8).

Information analysis of Fis-binding sites and their surrounding sequences has revealed previously unidentified sites adjacent to known ones (5). We observed that pairs of Fis sites are often separated by 7 or 11 bases in many genetic systems (5,9). These Fis sites often overlap the binding sites of other proteins in biologically significant places such as the Xis site of λ *att* (10), *dif*, *nrd*, *ndh* and the *fis* promoter (data not shown). To understand the significance of these pairs, we sought to determine whether Fis binds cooperatively or antagonistically at the adjacent sites. In this study, we show that in artificial DNA constructs, overlapping Fis sites 7 or 11 bp apart cannot be bound simultaneously and therefore act as a molecular flip–flop.

DNA replication starts at 84.6 minutes on the circular *E.coli* K-12 chromosome at a locus called *oriC* (11,12). Bidirectional replication starting at *oriC* (13,14) is completed in the terminus region half way around the chromosome (15). Replication is dependent on the DnaA protein, which binds at five sites in *oriC*. Using sequence walkers (10), we observed that there are likely to be two Fis sites 11 base pairs (bp) apart wedged precisely between two of the DnaA sites. We show that these sites are not bound simultaneously.

MATERIALS AND METHODS

Sequence analysis programs

Delila system programs were used for handling sequences and information calculations (10,16–21). Figures were generated automatically from raw GenBank data using Delila and UNIX script programs. Further information is available at http://www.lecb.ncifcrf.gov/~toms/.

Design of Fis binding experiments

Synthetic DNAs containing strong Fis sites separated by 11 and 7 bp were designed by selecting from the most frequent bases at each position in the Fis sequence logo (5). These were then merged with the same sequence shifted by 11 or 7 bp by comparing the $R_{iw}(b,l)$ values for various choices. [Note: the consensus sequence of the early model we used was TTTG(G/C)TCAAAATTTGA(G/C)CAAA which differs from that of the logo (22). The 0 and strongly conserved ±7 positions are underlined.] Five extra bases were added to the ends based on the natural sequences around the *hin* proximal and medial sites for the overlap 11 oligo, and the sequences around *cin* external and proximal sites were used for the overlap 7 oligo (5). The DNAs were made self-complementary (Fig. 4a and b). Sites separated by 23 bases were created starting with the 11 base-separated DNA and duplicating the central overlap region. A BamHI site was also inserted and the DNA was flanked by EcoRI sites (Fig. 4c). Oligos were synthesized with biotin on the 5′ end and gel purified (Oligos Etc., Wilsonville, OR). To ensure thorough annealing, they were heated to 90°C for 10 min, and slowly cooled to room temperature. The annealed products were electrophoresed through an 8% (w/v) polyacrylamide gel, and the bands corresponding to the linear

*To whom correspondence should be addressed at National Cancer Institute at Frederick, PO Box B, Building 469, Room 144, Frederick, MD 21702-1201, USA. Tel: +301 846 5581; Fax: +301 846 5598; Email: toms@ncifcrf.gov
Present addresses:
Paul N. Hengen, Applied Biosystems, 3833 North First Street, San Jose, CA, 95134-1701, USA
Lisa E. Stewart, Alkami Biosystems, Inc., PO Box 11216, Berkeley, CA 94712-2216, USA

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

duplex DNA of the correct size were sliced from the gel. DNA was recovered by electroelution and extracted with isoamyl alcohol to remove ethidium bromide. A non-specific control DNA was composed of the two 66 bp HinfI fragments from bacteriophage ϕX174 (Life Technologies, Inc.). This DNA did not shift even at the highest concentration of Fis used. Gel mobility shift experiments were performed as described previously, using chemiluminescent detection (5). Fis used in the experiment shown in Figure 5 was a gift from Reid Johnson.

Hairpin DNA oligos containing the *oriC* region (Fig. 7) were synthesized with a 5′-tetramethylrhodamine modification and gel purified (Oligos Etc.). By using a hairpin, an annealing step is not required and the oligos are exactly equimolar (5). Horizontal 8% PAGE was used for the gel mobility shift assay and band positions were visualized with a FMBIO II fluorescent scanner (Hitachi) with an excitation wavelength of 532 nm and detection at 605 nm.

Fis protein isolation

For the experiment shown in Figure 7, Epicurian Coli® BL21-Gold(DE3) cells (Stratagene) (23) transformed with pRJ1077 plasmid (a gift from Reid Johnson) were used for Fis protein isolation by modification of a previous method (24). A 10 ml overnight culture was added to 1 l of SOB medium, shaken at 37°C for 2 h 30 min, induced by 1 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and shaken again for 1 h. The culture was centrifuged and the cell pellet was resuspended in 50 ml of FLBC buffer (50 mM Tris–HCl pH 7.5, 10 mM EDTA), containing 200 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), sonicated with a Cole-Parmer Ultrasonic Processor model CP 70T three times for 10 min (40 W) on ice and centrifuged at 2000 *g* for 20 min. The supernatant was loaded on a 10 ml Q-Sepharose® Fast Flow (Amersham Pharmacia Biotech) column equilibrated with the same buffer and washed with 10 ml of FLBC buffer containing 300 mM NaCl. Flow-through and wash fractions were combined and the mix was loaded on a 10 ml heparin–Sepharose® CL-6B column (Amersham Pharmacia Biotech), washed with 50 ml of FLBC buffer containing 300 mM NaCl and eluted with a 100 ml 300–2000 mM NaCl gradient of FLBC buffer. Fractions were analyzed by SDS–PAGE.

Molecular models of Fis binding to DNA

In order to understand the sequence logo for Fis-binding sites (5), we constructed three-dimensional models of Fis DNA binding. The models of Fis interacting with DNA were built using Insight II software from Biosym Technologies, Inc., on an IRIS computer (Silicon Graphics, Inc.), and displayed with RasMol 2.5, available at http://molbiol.soton.ac.uk/rasmol.html or ftp://ftp.dcs.ed.ac.uk/pub/rasmol/. The Fis protein coordinates are those of the Protein Data Bank (http://www.rcsb.org/pdb/) entry 1fia.

In Fis–DNA models built by earlier workers, either B-form DNA with no particular base sequence was smoothly bent and placed next to the X-ray crystal model of Fis (25,26), or the single distal *hin* Fis-binding site (V01370, 180, 8.9 bits) was used and kinks added to the DNA at positions based on the DNase I-hypersensitive pattern (27). Since the sequence logo gives a detailed model for the sequence conservation at Fis sites, we used this information to construct three models: (i) a single Fis homodimer binding to the consensus sequence as determined by the logo (Fig. 3a); (ii) two Fis homodimers binding to two Fis sites spaced 11 bp apart on DNA (Fig. 3b); and (iii) two Fis homodimers binding to two Fis sites spaced 7 bp apart on DNA (Fig. 3c).

We used several criteria for making the first model. As noted by previous workers, Arg85 and Lys91 in the D helix are likely to be involved in DNA binding since mutations at these amino acids interfere with DNA binding (25,26). The logo shows highly conserved G–C base pairs at –7 and +7 in major groove positions, as is common when contacts are made by proteins with helix–turn–helix motifs (27,28). In addition, methylation of these bases blocks Fis from binding (29) and Fis protects these bases against methylation (30). However, as mentioned in previous studies, with B-form DNA, the Gs at ±7 would be further apart than the distance between the two DNA-contacting D helices of Fis. Fis can deflect DNA from 40 to 90° (2,25,27,31), so we can attain a shorter distance between the two DNA contact points at ±7 either by 'bending' (a gradual curvature of the helix axis over a sequence of more than two bases) or by 'kinking' (a sharp change in the DNA strand's axial direction between two adjacent bases) at one or more locations between the two contacts (32).

If specific contacts are not formed, a smoothly bent DNA might be expected to produce smooth sequence conservation over the binding region, but the logo shows strong variable conservation of base sequences at positions –5 through +5, so it is likely that the deflections are not smooth. Furthermore, both CA and TG, which are conserved at positions –4 to –3 and +3 to +4 (26) (i.e. positions ±3.5), have been shown to display unusually large roll [rotation about the long axis between base pairs (33)]. The CRP–DNA complex displays a 90° bend that results from CA and TG kinking (34,35) [see Papp *et al*. (28) for the CRP sequence logo].

Additionally, the other two pyrimidine–purine (Py–Pu) base sequences conserved in Fis sites, TA (36) and CG (37–40), show this high-roll characteristic. These four kinking dinucleotides appear frequently at ±3.5 and ±7.5. They account for the base frequencies in the logo and they identify kink locations which could result in the observed DNA deflection (41). Examination of the individual Fis-binding sites confirms this observation. With the exception of seven Fis sites in our set: *fis* X62399 292, *nrd* K02672 3266, *aldB* L40742 153, *hin* V01370 68, *cin* X01828 289, *gin* M10193 389 and *hns* X07688 655, sites which do not have a Py–Pu step at ±3.5, do have one displaced just one position left or right (34). In addition, these Py–Pu kinks form during energetic minimization of a Fis–DNA complex (42). We therefore constructed our model using two 42° kinks at ±3.5. More kinking would prevent Fis from fitting into the two successive major grooves. Two 50° kinks were also added at positions ±7.5 because this was the largest observed kink found for CAP protein binding (35).

The midsection of the logo is AT rich and this should create a higher twist, leading to a compression in the minor groove (26,32,43). We incorporated this compression into our model by making the helical repeat through the central A–T tract (bases –2 to +2) 10 bp per turn and setting the remainder of the model to 10.6 bp per turn for B-form DNA (44–46).

We tried to maximize the overall DNA bend angle of Fis bound to DNA and found that the best we could do, while

avoiding stereochemical collisions, was 60°. This model of a single Fis homodimer contacting DNA (Fig. 3a) has two kinks and one compression that allow the four nitrogens of Arg85 to be within contact distance (<3.5 Å) of either the O6 or N7 acceptor of each G at ±7 and Lys91 to be within 3.5 Å of the phosphate backbone at ±1.5 with no major structural conflicts.

To investigate the consequences of two Fis molecules binding to nearby sites, we constructed three-dimensional models with Fis sites separated by 11 or 7 bp. For the two overlapping Fis models, we extended the general scheme of modeling a Fis homodimer to the synthetic DNA sequences used in our gel shift experiment.

For the overlap 11 model (Fig. 3b), different kinks from the two adjacent sites would be in the same place. Because 50° kinks would prevent Fis from fitting into either site, we made both 42°. We incorporated 50° kinks on the outside two positions, as in the single site model. We also increased the twist to –36° at all A–T pairs within the central region (36).

For the overlap 7 model (Fig. 3c), the two outside TG and CA ends were kinked 50° to conform to the single Fis model. However, two pairs which would normally be kinked were not Py–Pu pairs in this sequence. We excluded kinking of the left site at ±7.5 due to a Py–Py pair (CT) and we excluded kinking of the right site at –7.5 due to a Pu–Pu pair (AG). Therefore, we only added 42° kinks at three positions: at two Py–Pu pairs (left –3.5 CA and right +3.5 TG) which correspond to the single Fis model, and at one in the center (TA). As in the overlap 11 model, twists of –36° were made throughout any A–T tract to be consistent with the single homodimer model.

We found that two Fis proteins separated by 11 bp would strongly interpenetrate. On the other hand, two Fis proteins separated by 7 bp have a minimal van der Waals force conflict between the two central D helices, but this might be accommodated for by flexibility of the DNA–protein complex. Given that the D helices cannot fit directly into B-form DNA, there is some uncertainty as to how Fis binds DNA. We thought that 11 base-separated Fis molecules (Fig. 3b) would compete for binding but that a seven base separation (Fig. 3c) might allow simultaneous binding.

RESULTS AND DISCUSSION

Fis site structures suggest self-competition

In a previous study, we used 60 experimentally defined Fis-binding sites to construct a highly detailed and reliable model of Fis binding to DNA (5). When we searched DNA sequences for Fis sites using this information-theory based weight matrix model, we observed Fis sites spaced 11 bp apart in the DNA inversion systems *hin*, *gin* and *min* [see fig. 5 in Hengen *et al*. (5)]. A search of the entire *E.coli* genome shows that Fis sites are frequently separated by 11 bases (Fig. 1). Although the same number of sites is found in a genome-sized equiprobable random sequence, this does not mean that the sites are not functional. Many binding sites have just the amount of information ($R_{sequence}$) needed for them to be found in the genome ($R_{frequency}$) (18). According to Shannon's principles (47), a well-coded communications system looks like random noise from the outside, and Fis site pairs may follow this principle. So the spike of sites coming in pairs is caused by the pattern of Fis itself; it is intrinsic to the Fis model. We cannot tell if the pattern evolved because Fis sites were required to overlap or whether Fis sites overlap because the pattern evolved. The peaks of Fis sites every three bases in the genomic scan come from protein coding effects. The peak of spacing at 8 bases and excess sites at spacings of 1 and 3 bases were only noticed during revisions of this manuscript; we do not understand their significance.

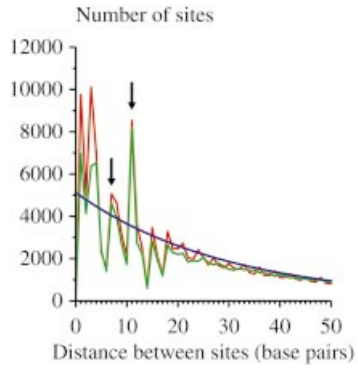

Figure 1. Predicted Fis site spacings. An information theory-based model of Fis protein DNA binding was scanned across the *E.coli* genome. The distances between the zero coordinates of successive sites were recorded and tabulated in this graph. Red curve, search of *E.coli* genome; green curve, search over equiprobable random sequence; blue curve, mathematical model for randomly placed sites. This model was constructed by considering a genome of size G = 4 639 221 bases (the U00096 *E.coli* K-12 MG1655 genome) having n = 154 112 sites ($R_i > 0$ bits) so that the probability of a site being at one position is $p = n/G$. Then the number of sites with separation d is $Gp^2(1 - p)^d$. Similar results are obtained for a 2.5 bit cut-off, the lowest observed Fis site in our set. Arrows indicate spacings of 7 and 11 bp.

Since B-form DNA twists every 10.6 bp, the sites, spaced 11 bp apart, should be on the same side of the DNA. While it is possible for two adjacent proteins to bind simultaneously by a subtle interleaving of their DNA contacts, as in the case of RNA polymerases (48,49), it seems more likely that in this case they will compete for binding in the major groove since, after an 11 base shift, the sequence logo shows that the predominant G at –7 corresponds to the G at +4 and the C at –4 corresponds to the C at +7 (red arrows in Fig. 2). Competition between these internally redundant patterns (9) would allow Fis to change the point at which the DNA is bent. Perhaps this is important for inversion.

In contrast to the 11 base spacing described above, in bacteriophage P1 *cin*, bacteriophage P7 *cin*, *E.coli e14 pin* sites, and in the genome scan (Fig. 1), pairs of Fis sites are observed at spacings of seven bases. This would place the Fis dimers 122° apart on B-DNA (360° – 360° per turn × 7 bases/10.6 bases per turn = 122°). After a seven base shift, the sequence logo shows that the predominant G at –7 would match the A/T/g/c of the minor groove on the opposite face of the DNA at coordinate 0, while the C/T at –4 would match the T/C at +3 and the A/G at –3 would match the G/A at +4 (green arrows in Fig. 2). This allows for the possibility that the two proteins bind at the same time, which might also be important to the function of these regions.

To investigate the consequences of two Fis molecules binding to nearby sites, we constructed three-dimensional

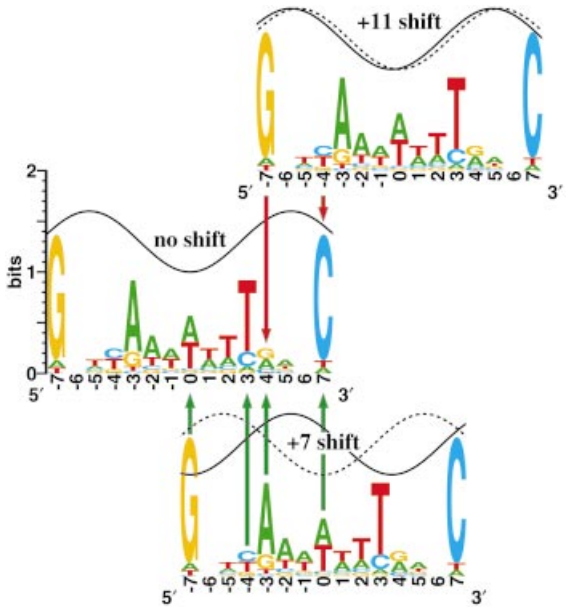

Figure 2. Self-similarity of Fis-binding sites. The sequence logo for Fis (5,19) is shown three times. The upper and lower logos are shifted +11 and +7 bases to the right (respectively) relative to the middle logo. Dashed waves indicate the phase of the shifted site; solid waves indicate the phase of the unshifted site. The in-phase sine waves, with a wavelength of 10.6 bases, show that Fis sites shifted by 11 bases would be on the same face of the DNA (28,101,102), while the out-of-phase waves of Fis sites shifted by seven bases indicate binding to opposite faces. Arrows are at positions where the logo is self-similar after a shift. Red arrows (pointing downwards from the +11 shift) mean that the contacts by Fis to the bases would interfere because they would be on the same face of the DNA. Green arrows (pointing upwards from the +7 shift) mean that the contacts could be simultaneous because they are on opposite faces. In a sequence logo, the height of each letter is proportional to the frequency of the corresponding base at that position in the sites, and the height of the stack of letters represents the sequence conservation in bits. For clarity, the sine waves run from 1 to 1.6 bits.

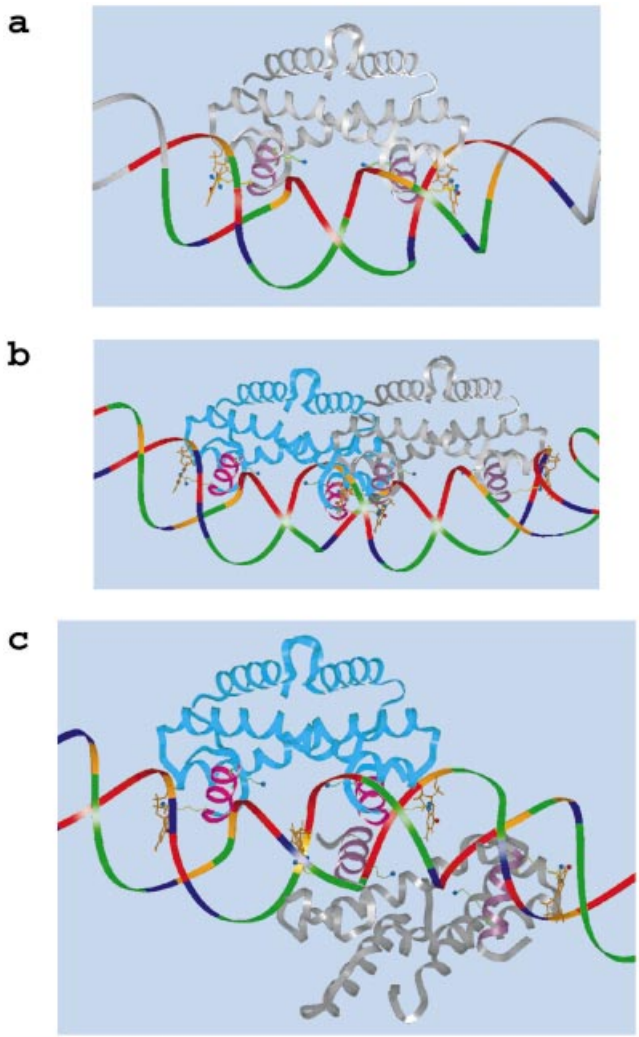

Figure 3. Fis binding models. (**a**) A single Fis dimer binding to DNA. (**b**) Two Fis dimers binding to Fis sites separated by 11 bp. (**c**) Two Fis dimers binding to Fis sites separated by 7 bp. The DNA backbone is color coded: A, green; C, blue; G, orange; and T, red. The models of Fis interacting with DNA were built using Insight II software from Biosym Technologies, Inc., on an IRIS computer (Silicon Graphics, Inc.), and displayed with RasMol 2.5, available at http://molbiol.soton.ac.uk/rasmol.html or ftp://ftp.dcs.ed.ac.uk/pub/rasmol/. The Fis protein coordinates are those of the Protein Data Bank (http://www.rcsb.org/pdb/) entry 1fia (see Materials and Methods for further details)

models (Fig. 3). We found that two Fis proteins bound to sites separated by 11 bp might strongly interpenetrate. In contrast, a 7 bp separation might only have a minimal van der Waals force conflict between the two central D helices. This might be accommodated for by flexibility of the DNA–protein complex, given that there is some uncertainty as to how Fis binds DNA. The models suggest that 11 base-separated Fis molecules would compete for binding but that a 7 base separation might allow simultaneous binding.

These ideas are supported by the preliminary observation that synthetic DNA containing either the *hin* proximal or medial Fis sites are bound by Fis in electrophoretic mobility gel shift assays (5). When these overlapping sites were together on the same fragment with a spacing of 11 bases, only one band shift was observed, suggesting that only one of the sites can be bound at a time. To test whether this is the case requires using high concentrations of Fis and strong Fis sites to ensure that both sites would be bound if that were possible.

Test of the self-competition model

To determine whether overlapping Fis sites can be simultaneously bound by Fis, we synthesized self-complementary DNA oligos with strong Fis sites that overlap by either 7 or 11 bp (Fig. 4a and b) or that were separated by 23 bp (Fig. 4c) and tested their properties by gel shift. Neither the 11 nor the 7 bp overlapping sites showed a doubly shifted band, even at an extremely high Fis/DNA ratio and with exceptionally strong (>12 bits) Fis-binding sites (Fig. 5), suggesting that only one Fis molecule could bind to each DNA fragment. The DNA fragment with two Fis sites separated by 23 bp did double shift (Fig. 5), demonstrating that two well separated Fis sites can simultaneously bind two Fis molecules. However, Fis can create a ladder of complexes on non-specific sequences (50), and this might account for the double shifts. Under our conditions with short DNAs, a non-specific (all positions <1 bit) 66 bp DNA fragment barely shifted at high Fis concentration (data not shown), so the secondary shifts were not from

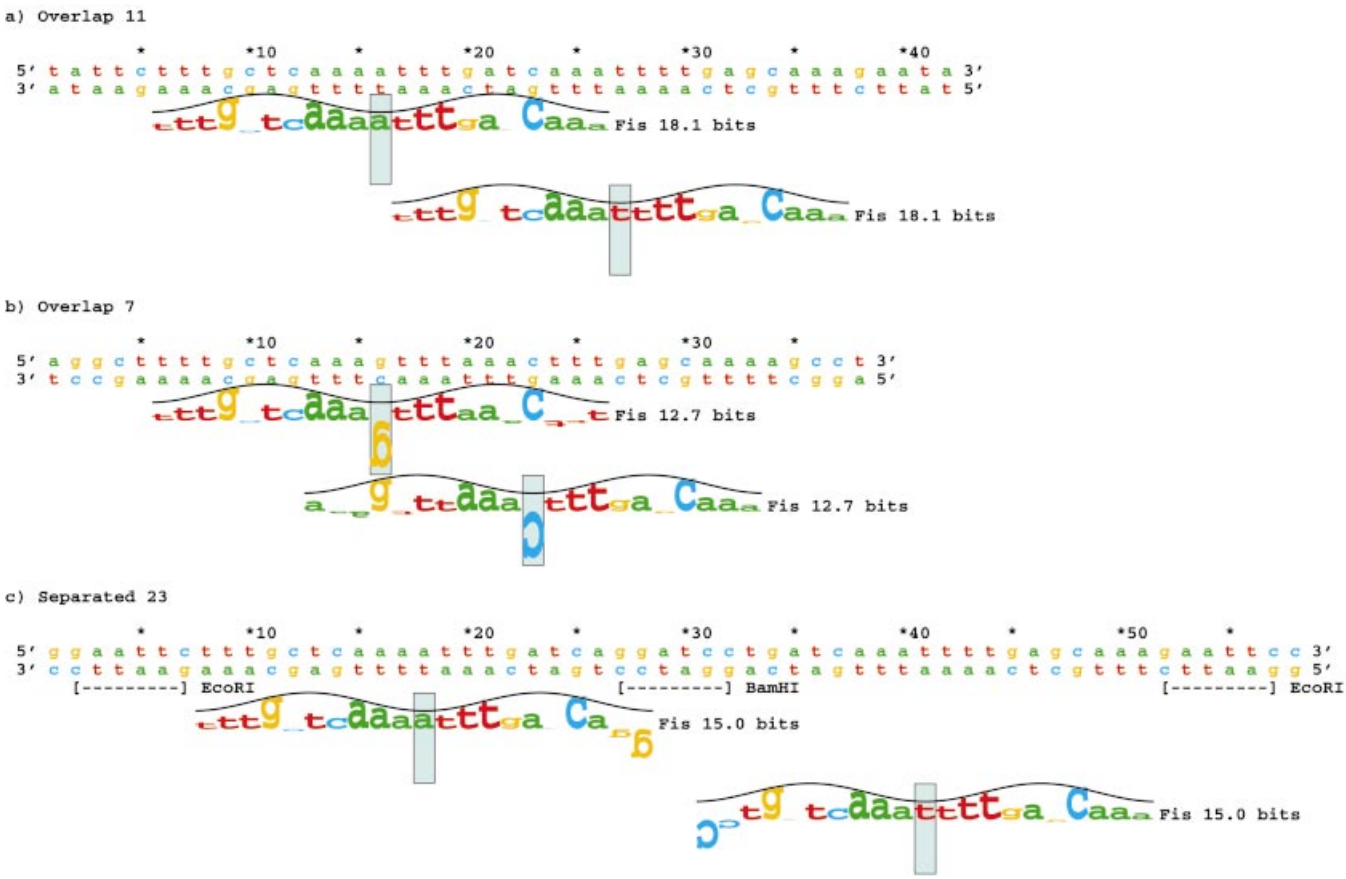

Figure 4. Oligonucleotide design of overlapping and separated Fis-binding sites. The predicted Fis sites are shown by sequence walkers floating below each self-complementary DNA sequence (5,10). In a walker, the vertical green box marks the zero base of the binding site. The box also shows the vertical scale, with the upper edge being at +2 bits and the lower edge being at –3 bits. The height of each letter is determined from the bit value in the individual information weight matrix (5,10,21). Negative weights are represented by drawing the letter upside down and placing it below the zero bit level. To indicate predicted relative orientations, the peaks of sine waves correspond to where Fis would bind into the major groove. Three DNAs were designed, each having two Fis sites spaced 11, 7 and 23 bases apart. Design details are given in Materials and Methods. The total strength of a site is the sum of the information weights for each base. The 18.1 bit Fis sites are 3.4 standard deviations higher than the average Fis site in natural sequences (5,21). The 12.7 and 15.0 bit sites are 1.6 and 2.4 standard deviations above average, respectively.

non-specific binding. These results demonstrate that Fis sites separated by 7 or 11 bases cannot be bound simultaneously.

Genetic implications of Fis flip–flops

The *E.coli tyrT* promoter has three Fis sites separated by 20 and 31 bp. This separation corresponds to our 23 bp separated control experiment (Figs 4 and 5), in which two Fis molecules bind independently. The separation in *tyrT* is sufficient for three Fis dimers to simultaneously position themselves on the same face of the DNA to cooperatively bind an αCTD subunit and activate transcription of stable RNA promoters (51). In addition to this activation mechanism, which is based on separated sites, Fis may also have evolved another control mechanism that uses overlapping sites.

When we scanned our Fis individual information model across various sequences, we discovered 7 and 11 spacings at DNA inversion regions, the *fis*, *nrd* and *ndh* promoters, and at *dif*, *E.coli oriC* and λ *att* (5,10). In the latter three systems, sequence walkers for Fis sites overlap binding sites of other proteins in biologically significant places, so we do not think that Fis sites appear at this spacing merely because of the internal redundancy of the site. Scanning with the Fis weight matrix also reveals two strong Fis sites previously identified in *oriC* at coordinates 202 and 213, although only one was thought to be bound (52–54). Footprinting data from three different groups show protection covering one, the other and both sites (Fig. 6).

We confirmed both *oriC* Fis sites individually by a gel mobility shift experiment (Fig. 7). Because of our previous work on Fis promoters (5), we knew that we could use sequence walkers (10) to engineer the sites. In this way, we could know that we had destroyed one site without affecting the other and without creating new sites (48). When both sites were mutated (oligo **nn**), no shift was observed until a high Fis concentration (1000 nM) was used. We take this concentration to represent non-specific binding for all four oligos used in the experiment. When the left (oligo **no**) or right (oligo **on**) Fis sites were mutated, only a single shifted band was observed. This confirms our prediction that both sites can bind Fis. In the **on** control, a high band appeared at the highest Fis concentration. From the lower concentration lanes, we know that there is one specific binding site and, apparently, at this

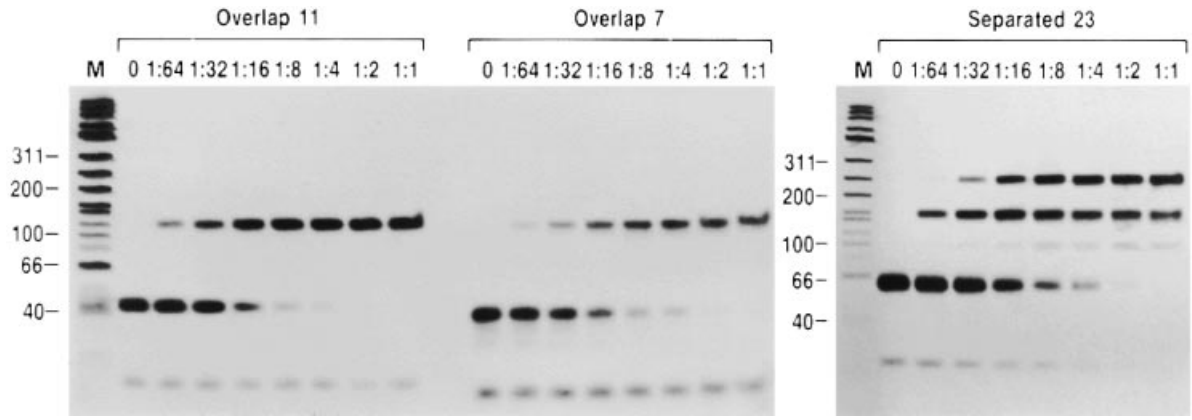

Figure 5. Mobility shift experiments for 11 and 7 bp overlapping and 23 bp separated Fis sites. Each lane contains increasing concentrations of Fis protein, beginning with no Fis, Fis diluted 1 to 64, etc. The 1:1 dilution was at 2200 nM Fis. This concentration was chosen intentionally so that with the 1 nM of DNA used in this experiment, the protein/DNA ratio was 2-fold higher than that needed to strongly shift DNA containing the 8.9 bit wild-type *hin* distal Fis site (29). The sequences are given in Figure 4. Marker lanes (M) contain 10 ng of biotinylated ϕX174 HinfI-digested DNA standards (Life Technologies, Inc.). Sizes are indicated in bp. The lowest band in most lanes of the figure is single-stranded oligonucleotide DNA. In the 'Separated 23' experiment, at high concentrations, Fis proteins are apparently able to capture the single-stranded DNA when it has folded into a hairpin. This produces a faint band near the 100 bp marker.

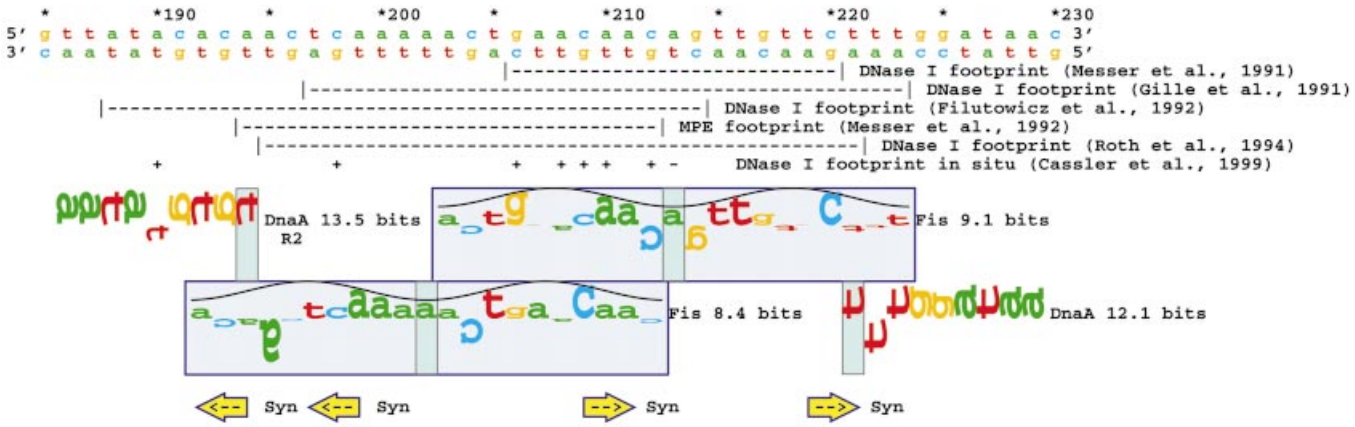

Figure 6. Positions of Fis and DnaA sites at the *E.coli oriC* shown by sequence walkers. Sequence data are from GenBank accession No. K01789 (103). The horizontal dashes below the sequence represent regions protected by Fis. Locations of DnaA sites are from Messer *et al.* (104) and Fis footprint data are from Gille *et al.* (52), Roth *et al.* (53), Messer *et al.* (54,104), Filutowicz *et al.* (66) and Cassler *et al.* (105). The asymmetric DnaA individual information matrix was created from 27 experimentally demonstrated DnaA-binding sites (102). DNA synthesis start sites are indicated by yellow arrows and 'Syn' (64); however, start sites have also been mapped to the left side of *oriC* (69). Blue boxes mark two Fis sites separated by 11 bases. DnaA site directionality is indicated by letters turned sideways in the direction that DnaA binds (10).

extremely high concentration a second Fis molecule binds non-specifically on this particular DNA sequence. This additional band may be explained by the existence of a number of weak (<0 bit) sites that can bind DNA at high Fis concentration. In any case, this band is absent in the experimental lane (**oo**).

The experiment also shows that the left Fis site, which is closer to DnaA R2 (oligo **on**, 8.4 bits) binds slightly less strongly than the one closer to DnaA R3 (oligo **no**, 9.1 bits), confirming the respective individual information contents, which differ by ~1 bit. When both sites were wild type (oligo **oo**), Fis binding was also observed but no supershift was visible. We conclude that only one Fis molecule can bind at a time between DnaA R2 and DnaA R3 of *oriC*.

The **oo** oligo can be bound in two distinct ways, so its association constant for Fis should be the sum of the two individual site association constants. This effect may have practical applications since creating overlapping binding sites will double the sensitivity of a biological detection system (55).

The two Fis sites at *oriC* fit exactly between the R2 and R3 DnaA sites and have similar individual information contents, suggesting that their binding energies are similar (56), so in the absence of other effects Fis could occupy them for nearly equal fractions of the time as a molecular flip–flop. The two states have not been recognized before because DNA footprinting only shows one predominant state or shows both states simultaneously, and such footprints have hitherto

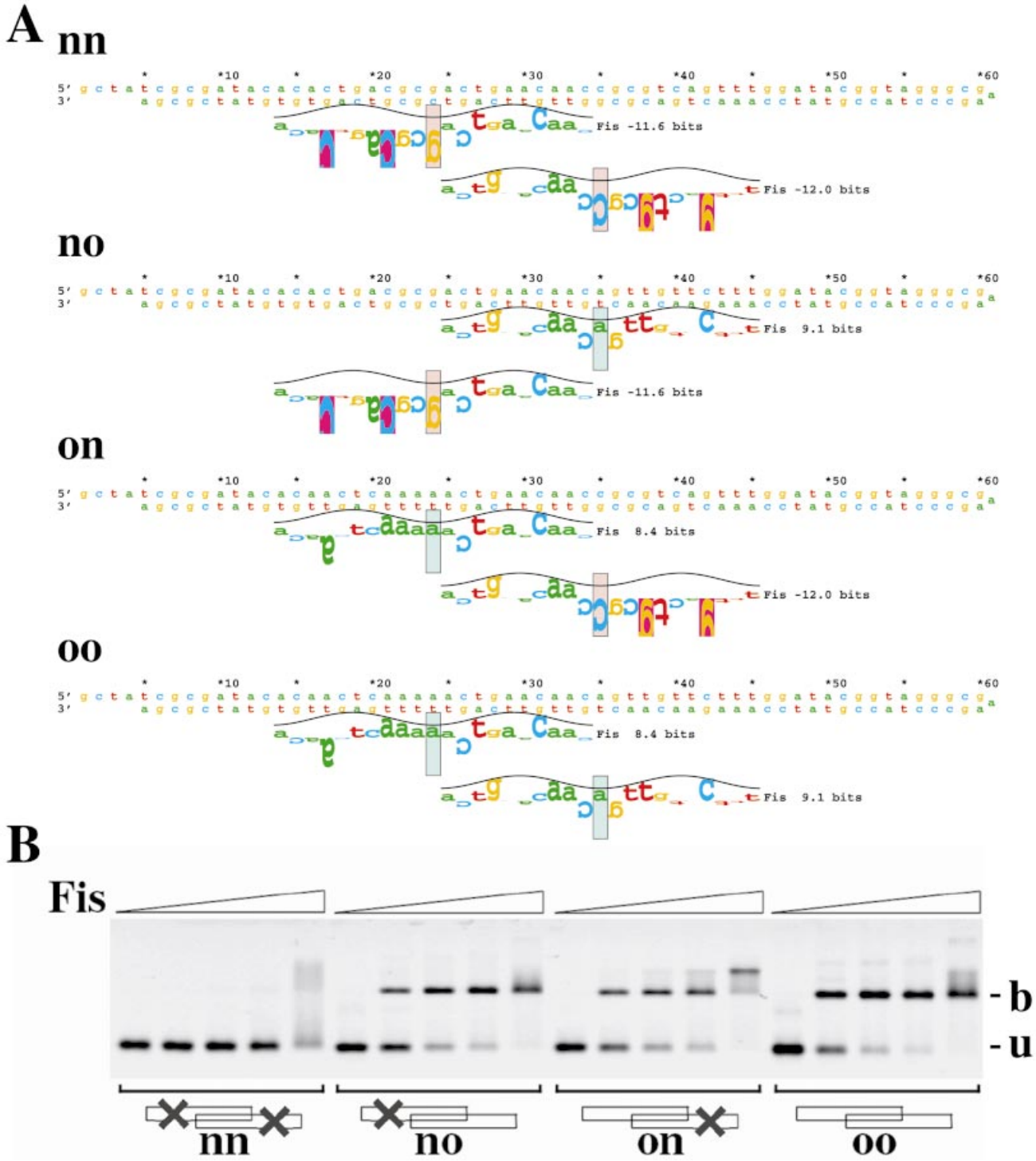

Figure 7. *E.coli oriC* can bind only one Fis molecule at a time between DnaA sites R2 and R3. (**A**) Design of wild-type and mutated Fis sites from *E.coli oriC*. Four hairpin oligos were designed and designated **nn**, **no**, **on** and **oo**, where **n** means no site because of engineered mutations (pink boxes, with information less than zero) and **o** means that there is a complete wild-type origin Fis site (green boxes, with positive information). For example, **no** contains only the Fis site closest to R3 on the right side. (**B**) Gel mobility shift assay with *oriC* sites using the oligos shown in (A) at a concentration of 10 nM each. Fis concentrations were 0, 30, 100, 300 and 1000 nM. u, unbound DNA; b, Fis-bound DNA.

been interpreted as representing single sites, i.e. the macroscopic experiments did not reveal that there are two distinct binding modes at dual Fis sites.

Our results resolve two previously conflicting reports. Gille *et al.* found that Fis binding and DnaA binding at R3 are mutually exclusive (52), but Margulies and Kaguni found that

they could bind concurrently (57). The controversy may be resolved by noting that different experimental techniques were used and that, because consensus sequences were being used, it was not clear that there are two Fis sites (22). The experiments by Gille *et al.* were DNase I footprints, which show protection of the entire R2–R3 region when Fis is bound prior to DnaA, as would be expected from a mixture of two states. The experiments by Margulies and Kaguni were footprints and gel shifts. If DnaA binds to R3, Fis might be blocked at position 213 but Fis could still bind at the other site at position 202. It is possible that both experiments produced valid data but for different states of the flip–flop.

If binding by DnaA and Fis are mutually exclusive (52), then the position of a Fis-induced DNA bend could be controlled by DnaA and the binding of DnaA could be controlled by Fis. During nutritional upshifts when there is a high Fis concentration (4), occupancy of one Fis site should ensure that only one DnaA site is available at a time. DnaA directs the loading of the DnaB helicase which, in turn, determines the orientation of the DNA polymerase or DnaG primase (58) and therefore the direction of replication (12,59–62). Since the Fis flip–flop probably controls which of the two oppositely oriented DnaA sites can be bound, it may control the alternative firing of replication complexes in opposite directions (63). This appears to be consistent with the divergent directions of DNA initiation observed in this region, as shown in Figure 6 (64). Indeed, the absence of Fis leads to asynchronous replication (65), and at high temperatures *fis* null mutants have been shown to form filamentous cells and have aberrant nucleoid segregation (66). Although Fis is pleiotropic, these and other observations (67,68) are consistent with Fis being required for proper replication initiation. However, initiation using purified components in the absence of Fis is bidirectional (59,69). One possibility is that in the absence of Fis, loading orientation is random (69) and that initiation *in vivo* fails unless the complexes are oppositely oriented. This should occur ~25% of the time, and indeed when initiating molecules were counted, only 36% formed SSB bubbles (59). Since there may be other explanations for these data, further experiments will be needed to determine how the Fis flip–flop is involved in initiation.

A general model for how DnaA is involved in origin replication has been proposed (70,71) in which the R1 DnaA site is thought to be involved in opening the adjacent AT-rich 13mer region. That model does not include the two competing Fis sites demonstrated in this study. To combine the models, one possibility is that the genetic structure at R2–R3 is only functional at nutritional upshifts when there is a high concentration of Fis in the cell (4). Under these circumstances, coordination of replication fork firing may be critical to start the first or subsequent rounds of replication correctly.

Are these proposals supported by mutations in the Fis site(s)? An experiment by Weigel *et al.* was intended to destroy 'the' Fis site between R2 and R3 (72); however, analysis by sequence walkers shows that the *oriC*131 AACTCAA to ATGTGTA mutation decreased the left Fis site (at coordinate 202) from 8.4 to 3.1 bits, while leaving the right site at 213 unchanged (analysis not shown). When placed in the *E.coli* chromosome, the mutant shows a moderate change of asynchrony of initiation by flow cytometry. Unfortunately, the mutation also created an 8.9 bit DnaA site which makes the experimental results difficult to interpret. [This 8.9 bit site also was created in earlier experiments (53).] In another experiment, replacing six bases between R2 and R3 by a BamHI site decreased *oriC*-dependent plasmid transformation by 57-fold (73). Sequence walker analysis (not shown) indicates that this mutation destroys both Fis sites (<2 bits) while leaving the R2 and R3 DnaA sites intact. A 10 bp insertion at coordinate 203 (presumably between bases 203 and 204) destroyed the MPE (methidiumpropyl-EDTA-Fe^{2+}) footprint of the Fis site at 202 but the site at 213 still showed an MPE footprint (54). This same insert reduced transformation frequency of an *oriC* plasmid into a *polA* strain. These results suggest that the Fis flip–flop is important for replication from *oriC*.

When does the flip–flop state change? *In vivo* footprinting shows that during the cell cycle, DnaA sites R1, R2 and R4 are bound, but R3 is not occupied (74). R3 becomes occupied at initiation of DNA replication (75). R3 is also bound by DnaA more weakly than R2 *in vitro* (76,77). This is consistent with the information measures which suggest that R3 is 13.5–12.1 = 1.4 bits or at least $2^{1.4}$ = 2.6-fold weaker (Fig. 6). In the absence of other processes, R3 should be bound less frequently. We suggest that for the majority of the cell cycle, DnaA bound to R2 blocks Fis at position 202, which allows Fis to bind at 213 and which, in turn, entirely blocks R3 from being bound by DnaA. Replication initiation may temporarily alter the flip–flop state, exposing R3. These data suggest the alternative hypothesis that the flip–flop is part of an on–off switch controlling initiation in the presence of Fis, especially during nutritional upshift (4).

Closely spaced sites are often bound cooperatively, as in the classical example of T4 gene 32 autogenous regulation (78), and even at overlapping LexA sites on opposite faces of the DNA (79,80). In contrast, Fis represents the unusual situation where a protein competes with itself by binding at overlapping positions. Self-occlusion has been observed in artificial constructs, where one ribosome is apparently blocked by the presence of another ribosome bound nearby (81), by polymerases at promoters (48,49,82,83) and in enzyme complexes (84). An interplay of factors may be typical of complex flip–flop mechanisms. For example, as many as five Fis sites are likely to be in λ *att*. Two of these are spaced 11 bp apart, with one of them overlapping an Xis site (10). Likewise, at the *E.coli dif* locus, where the XerC and XerD site-specific recombination proteins bind (85), one finds an overlapping set of seven weak Fis sites; three of these are separated by 11 bases (data not shown).

The positioning of Fis-binding sites relative to one another and to the binding sites of other proteins therefore appears to be key for the ability of Fis to perform many diverse functions. Fis has evolved a transcriptional activation mode in which sites are on the same face of the DNA and are sufficiently far apart to be bound simultaneously (51). Fis may also have specifically evolved to allow for two competitive binding modes. When the sites are on the same face of DNA (11 bp apart), a single Fis molecule could disengage and rebind to move the bend location between two possible places without changing the overall direction of the DNA. When sites are on nearly opposite faces (7 bp apart), shifting a Fis dimer molecule would cause the bend direction to change by 122°. How these cogs fit into the larger picture of pleiotropic Fis

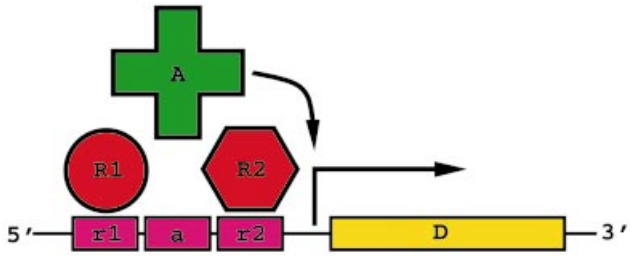

Figure 8. NOR gate molecular computer. An activator protein molecule **A** (green plus) binds to a DNA molecule at position **a**. When the activator binds, it turns on the promoter for gene D. Two repressor protein molecules **R1** and **R2** (red circle and red hexagon, respectively) bind to DNA at positions **r1** and **r2**. Binding to either **r1** or **r2** interferes with binding by **A**, so the activator can only bind when the two repressors are absent. Assigning the presence of a molecule as '1' or 'true' and the absence as '0' or 'false', then **D = R1 NOR R2**. By connecting such NOR gates together, any computer circuit can be built.

functions remains to be determined. However, an 11 bp shift of a bend would have dramatic effects on the *oriC* DNA initiation complex structure such as the ones suggested by Messer *et al.* (54,86).

Technological uses of molecular flip–flops

Molecular flip–flops such as the Fis flip–flop have at least two technological uses. First, binding equations for the flip–flop show that, compared with a single binding site, a dual-competitive site (in which both parts have the same binding energy) should have a doubled association constant (82). This curious property may be useful in biological assays, because it increases the sensitivity without increasing the amount of bound protein (55).

Secondly, the flip–flop and other exclusionary binding site clusters may be used to construct a molecular computer, since overlapping binding sites can provide Boolean logic (55). Previous molecular-based computers have used DNA hybridization and PCR (87), and carbon monoxide on copper surfaces (88) among other methods. Steps have been taken to evolve genetic circuits (89) and it has been demonstrated that the stability of gene networks can be increased by autoregulation (90) to provide sharp logical responses for 'digital genetics'. Genetic networks have also been shown to provide distinct toggle switching (91,92) and Boolean logic (93). In conjunction with these cell-sized chemical circuits, molecular flip–flops could be used to build molecular-scale circuits with sharp Boolean responses. A NOR gate can be constructed from a set of three binding sites, **r1**, **a** and **r2**, in which the middle site '**a**' is the binding site for a transcriptional activator **A** (55) (Fig. 8). When bound, **A** causes transcription of a downstream operon that can contain a gene for a signal such as GFP [green fluorescence protein (94)] or a DNA-binding protein for another part of the circuit. In the gate, **r1** overlaps **a**, and **r2** overlaps **a** from the other side. As shown in Figure 7, design of such constructs is facilitated by sequence walkers (10). If either the **R1**-or the **R2**-binding protein exists in the cell or solution, then the **A**-binding protein is excluded and the transcript is not expressed. Because this forms a NOR gate with amplification and fan-out at every step, any digital genetic circuit can be constructed, including complete computers (95–98). This method can take advantage of the inherent precision of protein–DNA recognition to provide low error rates (99,100). Unlike the gene-sized logic circuits previously described (93), which require about 3000 bases of DNA (1 μm), the functional component of these gates can be about 30 bases (10 nm) in length.

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