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Free Energy Contributions to Direct Readout of a DNA Sequence*

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The energetic contributions of individual DNA-contacting side chains to specific DNA recognition in the human papillomavirus 16 E2C-DNA complex is small (less than 1.0 kcal mol^{-1}), independent of the physical and chemical nature of the interaction, and is strictly additive. The sum of the individual contributions differs 1.0 kcal mol^{-1} from the binding energy of the wild-type protein. This difference corresponds to the contribution from the deformability of the DNA, known as "indirect readout." Thus, we can dissect the energetic contribution to DNA binding into 90% direct and 10% indirect readout components. The lack of high energy interactions indicates the absence of "hot spots," such as those found in protein-protein interfaces. These results are compatible with a highly dynamic and "wet" protein-DNA interface, yet highly specific and tight, where individual interactions are constantly being formed and broken.

A most fundamental issue in protein-DNA recognition is how a protein achieves a high degree of selectivity among different DNA sites of very similar overall structure, containing exquisitely fine differences in their chemical properties (1). The specific DNA site location must be kinetically accessible within biologically relevant time scales, given that the specific DNA binding site is immersed in a vast excess of potential nonspecific binding sites.

The energetic contributions to the binding of proteins to specific DNA sequences is the result of the formation of an intricate network of contacts between the binding partners, often coupled to conformational rearrangements in either or both macromolecules (2). Integrated structural and energetic analysis of protein-DNA interaction systems reveals that specific recognition involves direct contacts between the amino acid side chains and DNA bases, a mechanism known as "direct readout" of the DNA sequence (3). The lack of a simple correspondence between amino acid residues and specific DNA bases precludes the identification of a simple code for predicting the observed binding energetics (4). In addition, the fact that mutations on DNA bases that are not directly contacted by the protein often affect the interaction energetics (5) implies that local or distant sequence-dependent conformational transitions of the DNA molecule also affect protein-DNA binding, a mechanism termed indirect readout (6). Precisely how these two mechanisms contribute to the overall binding energetics is not known, and statistically based potentials predict that their relative contributions are not the same for all protein-DNA pairs (3). Experimental determination of the energetic contributions of direct and indirect readout requires modification of amino acid side chains and/or DNA bases, perturbations that would normally affect the structural and energetic properties of distant, non-targeted contacts (7, 8). These mutually dependent effects preclude a straightforward assignment of the energetic contributions to DNA binding in most protein-DNA systems.

The DNA binding domain of the papillomavirus E2 transcriptional regulator (E2C) is emerging as a remarkable model system to analyze these effects. E2 proteins bind at multiple sites on the viral and host genomes, regulate the expression of all viral genes, and participate in viral DNA replication (9, 10). The E2 binding sites of all viral strains consist of a highly conserved dodecameric sequence, ACCG*NNNN*CGGT, where *N* is any base separating the palindromic half-sites. Each half-site is symmetrically contacted by one DNA binding helix of each monomer, forming a discontinuous protein-DNA interface responsible for DNA binding specificity (11, 12). Mutations at the central non-contacted "spacer" sequence affect the binding affinity (13), and recent studies propose a partial structural code for specific site recognition (14). The E2C-DNA system is of particular interest because the interaction involves components of both direct and indirect readouts of the DNA sequence and, in contrast to many other protein-DNA systems, there is an established decoupling between the energetic contributions from both mechanisms (15). In the present study, we experimentally traced the origins of the direct readout energetics by means of a systematic rational perturbation approach of the DNA binding interface and determined that most of the binding energy comes from this recognition mode in this model system.

MATERIALS AND METHODS

Proteins—Wild-type HPV16 E2C mutants were produced, expressed, and purified as described previously (25).

Circular Dichroism—Samples containing 10 μM E2C proteins were extensively dialyzed against 10 mM sodium acetate buffer, pH 5.6, in 1 mM dithiothreitol, and far UV CD spectra were recorded using the Jasco J810 equipment. Thermal denaturation was followed by the change in molar ellipticity at 225 nm. The data were fitted to a standard concentration-dependent temperature denaturation equation (41).

Determination of the Equilibrium Dissociation Constants—Equilibrium binding of E2C protein to a specific E2 DNA site was carried out by fluorescence spectroscopy as described previously (25). Briefly, 0.5–50 nM solutions of a fluorescein-labeled DNA oligonucleotide containing a specific HPV16 E2 site in a buffer containing 20 mM bis-Tris[4]/HCl, pH 7.0, 1 mM dithiothreitol, and 0.2 M NaCl at 25 ± 0.1 °C were titrated with increasing amounts of the corresponding protein. The data were fitted to a simple quadratic binding equation, and the K_D and ΔG were calculated (25).

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[4] The abbreviation used is: bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

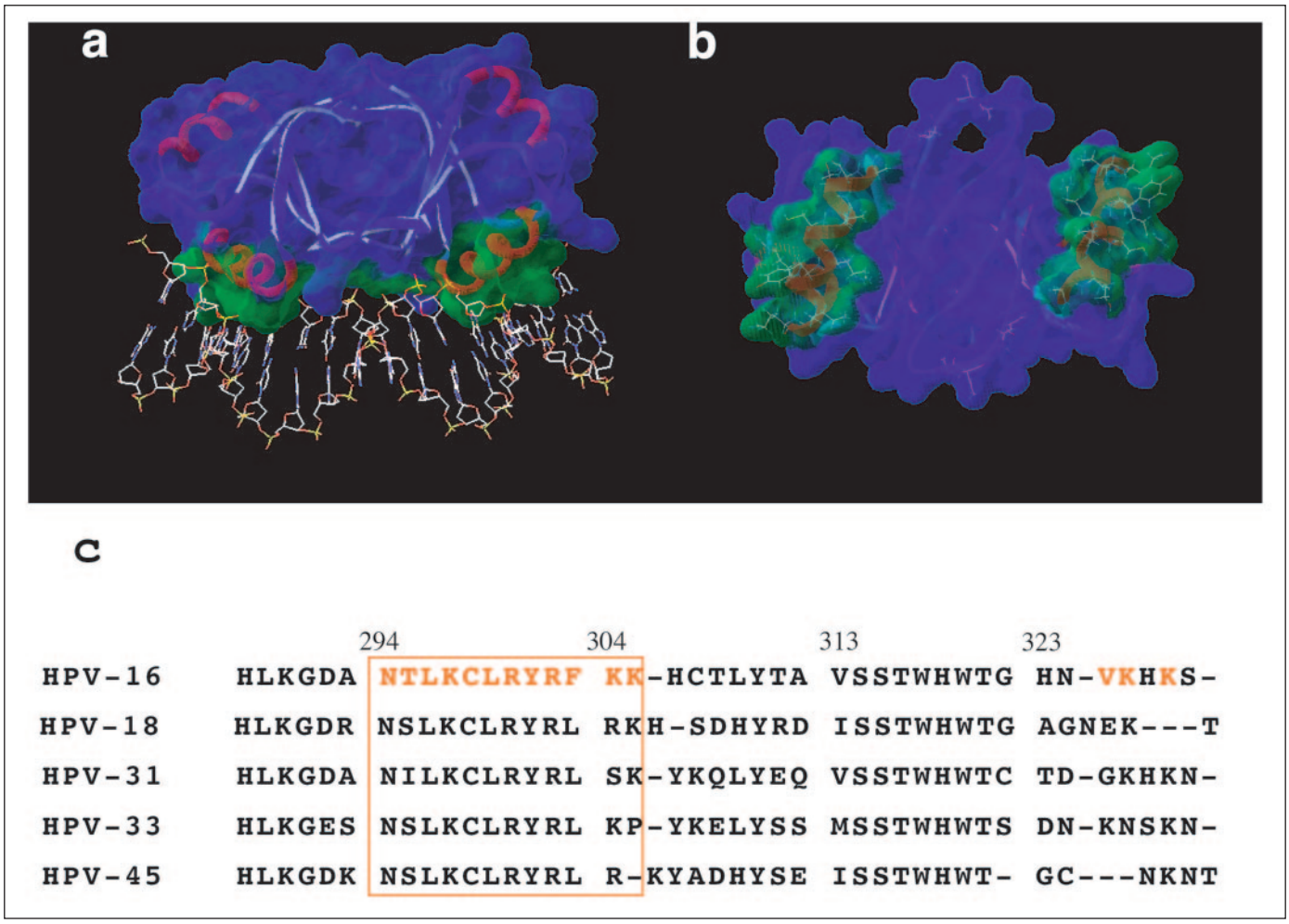

FIGURE 1. **The DNA binding interface of the E2C-DNA complex.** *a*, crystal structure of the HPV18 E2C domain bound to specific DNA (12) (Protein Data Bank accession code, 1JJ4). *b*, representation of the DNA binding helices of free HPV16 E2C (Protein Data Bank accession code, 1R8P). *c*, comparison of the amino acid sequences of the DNA binding helices of HPV16 E2C to HPV18 E2C and domains from other relevant strains. The known and presumed (β2–β3 loop) DNA contacting residues that were mutated are shown in *color*.

RESULTS

Experimental Design and Characterization of the E2C Mutants—The E2C domain is formed by the homodimerization of two identical polypeptide chains that cooperatively fold (16, 17) into a dimeric β-barrel structure (Fig. 1, *a* and *b*) (18, 19). The structures of E2C-DNA complexes show that the two chains interdigitate at the β-barrel and expose four α-helices, two of which insert into successive major grooves of B-DNA, contacting the ACCG half-sites (12, 20). The macromolecular interface formed by E2C and the DNA site is an intricate network of contacts with electrostatic interactions between the basic side chains of E2C and the DNA phosphate backbone being predominant and direct or water-mediated H-bonds between the side chains and the bases. The central non-contacted spacer sequence is bent toward the minor groove and is responsible for most of the indirect readout contribution observed in this system (15). To assess the energetic contributions of the nucleotide base to side-chain interactions, we tackled an energetic dissection of the interface contacts by modifying each of the protein side chains by a rational site-directed mutagenesis approach (21).

The E2C domain of the HPV16 viral strain was chosen for this study because it is well characterized biophysically (16, 17, 22, 23), and there are extensive data on its DNA binding mechanism (22, 24–29). Interestingly, the binding affinity of HPV16 E2C has a stronger dependence on the spacer sequence of its DNA site when compared with its homologues, which suggests that it displays a maximal decoupling between direct and indirect readout effects (15). We mutated each contact residue of the DNA binding helix, from Asn-294 to Lys-305, covering the complete binding interface identified in the structure of the HPV18 DNA homologue (Fig. 1*c*). In addition, we probed the loop connecting β3 and β4, which was shown to contact the DNA backbone in the DNA-E2C (BPV1) homologue (20) but appears flexible in HPV strains (11), by carrying out five single mutations.

Direct Readout Energetics—For a mutagenesis perturbation approach to be interpretable it is essential to carefully test the integrity of the protein in terms of its overall structure and stability. We found that the designed mutations disrupt neither the overall structure of the E2C dimer nor the folding properties described for the wild-type protein, as judged by far-UV circular dichroism spectroscopy (Fig. 2, *inset B*). Moreover, all mutations analyzed were stable and cooperatively folded (Fig. 2, *inset A*).

DNA binding activity of the wild type and each of the mutants was monitored by a fluorescence spectroscopy assay in solution. This allowed us to keep a minimal buffer composition in a well defined chemical environment and detect nanomolar binding affinities accurately (25). Because the mutant proteins bind to the same target oligonucleotide, the energetic differences can be attributed to the substitutions on the protein side chains, canceling out other effects. The change in binding free energy upon mutation ($\Delta\Delta G_{mut}$) is defined as

$$\Delta\Delta G_{mut} = \Delta G_{mut} - \Delta G_{wt} = -RT \ln(K_{Dmut}/K_{Dwt}) \quad \text{(Eq. 1)}$$

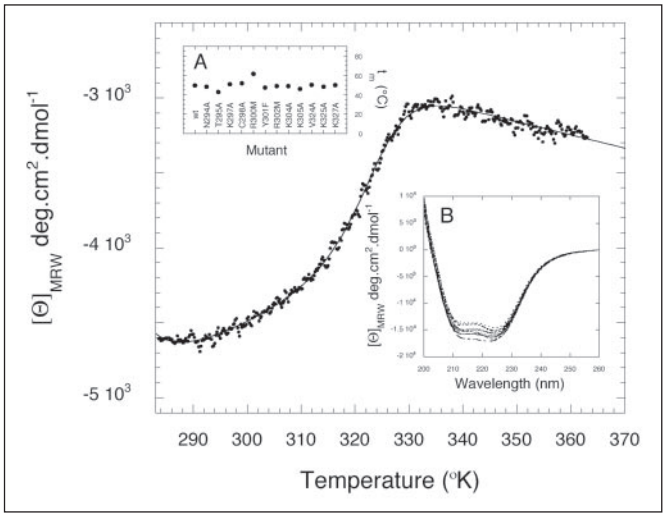

FIGURE 2. **Conservation of overall structure and stability of the E2C mutants.** Thermal denaturation of E2C variants was monitored by ellipticity changes at 225 nm (See "Materials and Methods"). *Inset A*, shows the calculated T_m for wild type and all mutants tested. *Inset B*, shows the superposition of the far-UV CD spectra of the proteins prior to thermal denaturation.

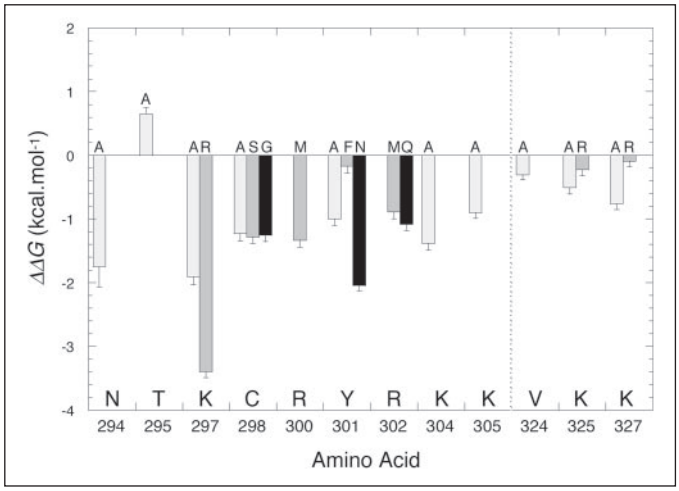

FIGURE 3. **Binding free energies for E2C proteins.** The $\Delta\Delta G$ for each mutant was calculated as explained under "Materials and Methods." The *vertical line* separates the distance in sequence mutants probing the $\beta2$–$\beta3$ loop.

TABLE ONE

Energetics of single side chain substitutions on binding of E2C to its specific DNA target

Mutation	K_D	$\Delta\Delta G^a$
	nM	*kcal mol^{-1}*
WT	1.20 ± 0.1	0
N294A	23.1 ± 11	**−1.75 ± 0.3**
T295A	0.30 ± 0.05	**+0.82 ± 0.12**
K297A	29.4 ± 5	**−1.89 ± 0.12**
K297R	335 ± 54	−3.33 ± 0.12
C298S	10.1 ± 1	−1.25 ± 0.1
C298G	10.5 ± 1.2	−1.28 ± 0.1
C298A	9.5 ± 1	**−1.22 ± 0.1**
R300M	11.4 ± 1.4	**−1.33 ± 0.1**
Y301N	38.0 ± 2	−2.04 ± 0.08
Y301F	1.58 ± 0.2	−0.16 ± 0.09
Y301A	6.5 ± 0.7	**−1.00 ± 0.1**
R302Q	7.5 ± 1.3	−1.08 ± 0.13
R302M	5.4 ± 0.3	**−0.89 ± 0.08**
K304A	12.4 ± 1.5	**−1.38 ± 0.1**
K305A	5.5 ± 0.3	**−0.90 ± 0.08**
V324A	1.6 ± 0.1	**−0.17 ± 0.09**
K325A	3.5 ± 0.3	**−0.63 ± 0.09**
K325R	1.04 ± 0.3	+0.08 ± 0.15
K327A	4.3 ± 0.2	**−0.75 ± 0.08**
K327R	1.4 ± 0.1	−0.091 ± 0.09
Total[b]		**−11.1 ± 0.5**

[a] Obtained by subtracting the ΔG of the mutants from that of the wild type (WT), 12.1 ± 0.08 kcal mol^{-1}. A negative sign indicates weaker binding.
[b] The sum of the most conserved substitutions for each side chain contacting the DNA; these substitutions are highlighted in bold.

where $K_{D\text{mut}}$ and $K_{D\text{wt}}$ are the dissociation constants between the oligonucleotide Fl site 35 and the mutant or wild-type protein, respectively. The results are shown in Fig. 3 and TABLE ONE.

In general, the energetic perturbation caused by the conservative mutations is small, between 0 and 2.0 kcal mol^{-1}, which means less than 1.0 kcal mol^{-1} assuming a symmetric energetic contribution by each mutated residue per monomer.

In addition, all but one of the mutants show a decrease in binding free energy in the range of the values assigned for similar substitutions in other protein-DNA systems, in line with single side-chain deletion effects (30, 31). Because our mutational strategy covers more than 95% of the interaction surface and in the absence of any single high energy interaction, we can confidently state that there is no hot spot in this protein-DNA binding interface, such as those described in protein-protein and other protein-DNA interfaces (32–34). Thus, it is safe to assume that the effects of the mutations are local, *i.e.* the overall structure and stability of the protein-DNA complex is not affected beyond the mutation site.

Analysis of Individual Side-chain Interactions—To assign energetic contributions to specific functional groups and validate the mutational strategy at the structural level, particular amino acid side chains were replaced with more than one substitution. Crystallographic data suggested that the -OH motif of Tyr-301 is not involved in direct interactions (12, 20). Mutation of this residue to Phe has minimal effect in the binding free energy, in agreement with the structural data. The replacement of the same residue with Ala allows us to estimate a contribution of ~1.0 kcal mol^{-1} to the interaction with the phenyl group, which is involved in stacking interactions with DNA bases (11, 12). Replacement of Cys-298 with either Ser, Ala, or Gly causes the same decrease in binding energy, indicating that the interactions that this residue makes cannot be replaced by its structural homologue Ser and that the conformational restrictions around the Cys-298 ϕ-ψ backbone angles do not play a significant role in DNA recognition.

It is not clear whether the loop connecting strands 2 and 3 ($\beta2$–$\beta3$ loop) participates in DNA binding, because it faces the "non-contacting spacer" region of the E2 site (12), and it is apparently too flexible to be observed in the crystal structures of the different HPV E2C variants obtained so far (18, 19). Our mutagenesis approach allows us to conclude that the positively charged Lys-325 and Lys-327 residues in the $\beta2$–$\beta3$ loop do participate in coulombic interactions, presumably with the DNA phosphate backbone, because replacement of both with Ala causes a decrease in binding free energy of ~0.5 to ~1.0 kcal mol^{-1}. This decrease is completely restored if the residues are replaced by Arg, suggesting that these residues participate in a nonspecific contact with the DNA backbone, which lacks strong geometrical constraints. Conversely, this compensatory effect is not observed in the Lys to Arg substitution made at the center of the binding helix, where the mutation K297R severely affects the binding affinity, indicating a strong steric hindrance (TABLE ONE). Residue Val-324 of the $\beta2$–$\beta3$ loop does not contribute to the binding energy.

TABLE TWO

Effects of double side-chain substitutions on E2C-DNA binding

Mutation	Predicted $\Delta\Delta G_{i+j}$[a]	Measured $\Delta\Delta G_{i+j}$[b]
	kcal mol^{-1}	*kcal mol*$^{-1}$
C298A + R300M	−2.55 ± 0.16	−2.52 ± 0.50
C298A + N294A	−2.97 ± 0.34	−2.93 ± 0.19
Y301A + N294A	−2.75 ± 0.32	−2.76 ± 0.41
C298A + K297A	−3.11 ± 0.16	−3.18 ± 0.15
Y301A + K297A	−2.89 ± 0.16	−3.26 ± 0.49

[a] As predicted by the sum of the effects of the individual substitutions, shown in TABLE ONE.
[b] Measured relative to the wild-type protein.

Lys-304 interacts with phosphate groups, contributing 1.4 kcal mol^{-1} to binding energy. Two other residues that contact the phosphate backbone are Thr-295 and Arg-300 (12). T205A is the only replacement that showed a mild albeit significant increase in affinity (0.8 kcal mol^{-1}, 0.4 kcal mol^{-1} per symmetric single interaction) that could be explained by less favorable interactions made by the Thr -OH group in the wild-type protein, but a double mutation or fine structural data would be required to confirm it. Replacement of Arg-300 by Met causes a decrease in binding energy of 1.3 kcal mol^{-1}, most likely resulting from the contribution of an electrostatic interaction with a DNA backbone phosphate.

The energetic values measured for each group are small, in the order of the thermal energy at room temperature (~0.6 kcal mol^{-1}). This suggests that the protein-DNA interface presents a highly dynamic character at physiological temperatures. For some protein-protein interaction systems, the energetic effect on point substitutions correlates with structural parameters, such as the change in solvent-accessible surface area (33). We found a poor correlation between the observed $\Delta\Delta G$ and the change in solvent-accessible surface area, molecular surface, or the number of contacts deleted by mutation (not shown).

The Energetic Contribution of Side Chains Is Strictly Additive—For the analysis of the DNA binding interface, we interpreted the effects of mutation on a strictly local basis, and as such, the observed energetic differences are likely to be additive (8, 32). This is also an assumption of most of the theoretical models of protein-DNA interaction developed to date (35, 36). To confirm our experimental results, we engineered E2C proteins carrying two substitutions and measured the change in binding affinity compared with the individual single mutations, thus constructing a double mutant cycle (21). If the effects of deletion of side chains are independent, the observed change in free energy for the double mutant $i+j$ should be equal to the sum of the independent mutations,

$$\Delta\Delta G_{i+j} = \Delta G_{i+j} - \Delta G_{wt} \quad \text{(Eq. 2)}$$

$$\Delta\Delta G_{i+j} = \Delta\Delta G_i + \Delta\Delta G_j \quad \text{(Eq. 3)}$$

where $\Delta\Delta G_{i,j}$ are the difference in free energy changes associated with the corresponding single amino acid substitutions. The results for five double mutants are shown in TABLE TWO. The additive nature of side-chain contributions to DNA binding is evident in all cases, which include the combination of polar, charged, or hydrophobic interactions, even for residues that are close in sequence. These results validate the underlying assumption and allow us to extend and analyze the overall energetic contributions of site-specific recognition by E2C.

If the effects of direct and indirect readout mechanisms are independent, the net free energy change on binding (ΔG_b) can be decomposed into

$$\Delta G_b \sim \Delta G_{dir} + \Delta G_{ind} \quad \text{(Eq. 4)}$$

where ΔG_{dir} and ΔG_{ind} are the free energies attributed to each mechanism. Moreover, if the direct readout contributions are additive, ΔG_{dir} can be estimated by the sum of all individual contacting mutations. Therefore, we can estimate that the overall contribution from the direct readout mechanism will be −11.1 ± 0.5 kcal mol^{-1} (TABLE ONE). The free overall binding energy, obtained from the measured association constant of wild-type E2C, is −12.1 ± 0.08 kcal mol^{-1}. Thus the binding process is 90% direct readout, and we assign the 1.0 kcal mol^{-1} difference to indirect readout, where the "bendability" of the spacer sequence and adaptation to the irregular binding surface are reported to facilitate DNA binding by E2 proteins (11, 12). Moreover, using a combined theoretical and experimental approach using cyclic DNA molecules and gel-shift binding assays, Zhang *et al.* (15) determined recently that the indirect readout contribution in this system lies between 1.0 and 3.0 kcal mol^{-1}.

DISCUSSION

The E2C-DNA complex is an excellent protein-DNA interaction model where *a priori* chosen mechanistic modes of interaction, indirect and direct readout, can be dissected. Mutations of the DNA bases "read" by the protein often lead to non-local conformational changes in the DNA structure that ultimately affect the binding affinity (37). To avoid this problem, we perturbed the protein-DNA interface by changing the protein side chains that build up this interface. By carrying out direct and accurate experimental measurements in solution, we were able to quantify the contribution of individual groups to binding free energy. The contribution is small, irrespective of the nature of the interfacial bond, *i.e.* coulombic, hydrogen bond, or Van der Waals.

We determined that at least 90% of the binding energy in this system comes from the direct readout of the DNA sequence and the rest from indirect readout. We showed that the local energetic perturbations behave in an additive manner and confirmed it by a series of double mutant cycles, without any single side-chain interface interaction accounting for drastic changes in binding energy as found in protein-protein interface hot spots (32). The absolute free energy change assigned to each contact is small, near the reference value of the kinetic energy available at physiological temperatures. We interpret the observed effects as small perturbations in a complex interaction network that is robust and flexible enough to tolerate point modifications of its constitutive elements. The emerging picture for this system is of a highly solvated and dynamic interface.

Water-mediated interactions appear to be as important as direct hydrogen bonds for the establishment of stability and specificity in molecular recognition; however, this is not a straightforward rationale (38). The number of interface waters from crystallographic and solvent perturbation studies is probably underestimated (29, 38). The complex between the E2C domain and DNA is a paradigmatic example for a wet protein-DNA interface as opposed to a "dry" interface represented by the TATA box-binding protein (38). The BPV1 E2C-DNA complex was shown to have 42 water molecules mediating protein-DNA interactions (20, 39).

Although the exclusion of bulk solvent is a late but essential step in the kinetic binding mechanism of E2C to specific DNA and to a lesser extent for nonspecific DNA (26), a substantial number of water molecules remain at this model interface, and this appears to be essential for its recognition mechanism. It remains to be established how different categories of water molecules playing different roles in specificity, stability, and dynamics participate at the protein-DNA interface. It appears plausible that water molecules are constantly filling gaps in this interface, where the different bonds

are constantly being formed and broken. Water itself could help rearrange spaces left by absent interacting groups with a minimum energetic penalty, something that is not easy to picture in a dry interface, whether it is a protein-protein or protein-DNA interface.

DNA recognition by the E2C domain in papillomaviruses is at the center of transcription control and the initiation of viral DNA replication. Related to this particular system, we were able to establish that a flexible loop participates in nonspecific electrostatic interactions with the DNA backbone, something so far not observed because of the lack of crystallographic densities around this region. In addition, a conserved Cys residue, unusual at DNA binding interfaces (20), appears not to be taking place in hydrogen bonding; rather, the hydrophobic nature of the sulfur atom appears to be participating in a key interaction. NMR studies of the E2C-DNA complex will be essential for integrating structural, mutagenesis, thermodynamic, kinetic, and dynamic studies. Finally, the binding energy values measured for side chains of different chemical properties and the interpretation of multiple replacements at a single residue will hopefully contribute to the understanding of the role of non-covalent interactions in protein-DNA recognition (40) and provide absolute energetic parameters that may be used in theoretical approaches.

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REFERENCES

1. von Hippel, P. H., and Berg, O. G. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1608–1612
2. Spolar, R. S., and Record, M. T., Jr. (1994) *Science* **263,** 777–784
3. Gromiha, M., Siebers, J. G., Selvaraj, S., Kono, H., and Sarai, A. (2004) *J. Mol. Biol.* **337,** 285–294
4. Matthews, B. W. (1988) *Nature* **335,** 294–295
5. Sarai, A., Mazur, J., Nussinov, R., and Jernigan, R. L. (1989) *Biochemistry* **28,** 7842–7849
6. Harrington, R. E., and Winicov, I. (1994) *Prog. Nucleic Acid Res. Mol. Biol.* **47,** 195–270
7. Engler, L. E., Sapienza, P., Dorner, L. F., Kucera, R., Schildkraut, I., and Jen-Jacobson, L. (2001) *J. Mol. Biol.* **307,** 619–636
8. Jen-Jacobson, L., Engler, L. E., and Jacobson, L. A. (2000) *Structure Fold. Des.* **8,** 1015–1023
9. McBride, A. A., Romanczuk, H., and Howley, P. M. (1991) *J. Biol. Chem.* **266,** 18411–18414
10. Ham, J., Dostatni, N., Gauthier, J. M., and Yaniv, M. (1991) *Trends Biochem. Sci.* **16,** 440–444
11. Hegde, R. S. (2002) *Annu. Rev. Biophys. Biomol. Struct.* **31,** 343–360
12. Kim, S. S., Tam, J. K., Wang, A. F., and Hegde, R. S. (2000) *J. Biol. Chem.* **275,** 31245–31254
13. Hines, C. S., Meghoo, C., Shetty, S., Biburger, M., Brenowitz, M., and Hegde, R. S. (1998) *J. Mol. Biol.* **276,** 809–818
14. Rozenberg, H., Rabinovich, D., Frolow, F., Hegde, R. S., and Shakked, Z. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15194–15199
15. Zhang, Y., Xi, Z., Hegde, R. S., Shakked, Z., and Crothers, D. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 8337–8341
16. Mok, Y. K., de Prat Gay, G., Butler, P. J., and Bycroft, M. (1996) *Protein Sci.* **5,** 310–319
17. Mok, Y. K., Bycroft, M., and de Prat-Gay, G. (1996) *Nat. Struct. Biol.* **3,** 711–717
18. Liang, H., Petros, A. M., Meadows, R. P., Yoon, H. S., Egan, D. A., Walter, K., Holzman, T. F., Robins, T., and Fesik, S. W. (1996) *Biochemistry* **35,** 2095–2103
19. Nadra, A. D., Eliseo, T., Mok, Y. K., Almeida, C. L., Bycroft, M., Paci, M., de Prat-Gay, G., and Cicero, D. O. (2004) *J. Biomol. NMR* **30,** 211–214
20. Hegde, R. S., Grossman, S. R., Laimins, L. A., and Sigler, P. B. (1992) *Nature* **359,** 505–512
21. Fersht, A. (1999) *Structure and Mechanism in Protein Science*, pp. 129–131, W. H. Freeman & Company, New York
22. Lima, L. M., and de Prat-Gay, G. (1997) *J. Biol. Chem.* **272,** 19295–19303
23. Mok, Y. K., Alonso, L. G., Lima, L. M., Bycroft, M., and de Prat-Gay, G. (2000) *Protein Sci.* **9,** 799–811
24. Sanders, C. M., and Maitland, N. J. (1994) *Nucleic Acids Res.* **22,** 4890–4897
25. Ferreiro, D. U., Lima, L. M., Nadra, A. D., Alonso, L. G., Goldbaum, F. A., and de Prat-Gay, G. (2000) *Biochemistry* **39,** 14692–14701
26. Ferreiro, D. U., and de Prat-Gay, G. (2003) *J. Mol. Biol.* **331,** 89–99
27. Alexander, K. A., and Phelps, W. C. (1996) *Biochemistry* **35,** 9864–9872
28. Dell, G., Wilkinson, K. W., Tranter, R., Parish, J., Brady, R. L., and Gaston, K. (2003) *J. Mol. Biol.* **334,** 979–991
29. Lima, L. M., and Silva, J. L. (2004) *J. Biol. Chem.* **279,** 47968–47974
30. Oobatake, M., Kono, H., Wang, Y., and Sarai, A. (2003) *Proteins* **53,** 33–43
31. Jen-Jacobson, L. (1997) *Biopolymers* **44,** 153–180
32. Wells, J. A. (1991) *Methods Enzymol.* **202,** 390–411
33. Bogan, A. A., and Thorn, K. S. (1998) *J. Mol. Biol.* **280,** 1–9
34. Anderson, E. M., Halsey, W. A., and Wuttke, D. S. (2003) *Biochemistry* **42,** 3751–3758
35. Selvaraj, S., Kono, H., and Sarai, A. (2002) *J. Mol. Biol.* **322,** 907–915
36. Luscombe, N. M., and Thornton, J. M. (2002) *J. Mol. Biol.* **320,** 991–1009
37. Kurpiewski, M. R., Engler, L. E., Wozniak, L. A., Kobylanska, A., Koziolkiewicz, M., Stec, W. J., and Jen-Jacobson, L. (2004) *Structure (Camb.)* **12,** 1775–1788
38. Janin, J. (1999) *Structure Fold. Des.* **7,** R277–R279
39. Levy, Y., and Onuchic, J. N. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 3325–3326
40. Pabo, C. O., and Nekludova, L. (2000) *J. Mol. Biol.* **301,** 597–624
41. Ruiz-Sanz, J., de Prat Gay, G., Otzen, D. E., and Fersht, A. R. (1995) *Biochemistry* **34,** 1695–1701