Common patterns in type II restriction enzyme binding sites

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ABSTRACT

Restriction enzymes are among the best studied examples of DNA binding proteins. In order to find general patterns in DNA recognition sites, which may reflect important properties of protein–DNA interaction, we analyse the binding sites of all known type II restriction endonucleases. We find a significantly enhanced GC content and discuss three explanations for this phenomenon. Moreover, we study patterns of nucleotide order in recognition sites. Our analysis reveals a striking accumulation of adjacent purines (R) or pyrimidines (Y). We discuss three possible reasons: RR/YY dinucleotides are characterized by (i) stronger H-bond donor and acceptor clusters, (ii) specific geometrical properties and (iii) a low stacking energy. These features make RR/YY steps particularly accessible for specific protein–DNA interactions. Finally, we show that the recognition sites of type II restriction enzymes are underrepresented in host genomes and in phage genomes.

INTRODUCTION

Protein–DNA interactions play a fundamental role in cell biology. For instance, the highly specific interactions between transcription factors and DNA are essential for proper gene expression regulation (1). The ‘immune system’ of bacteria and archaea relies on restriction endonucleases (REases) recognizing short sequences in foreign DNA with remarkable specificity and cleaving the target on both strands (2–4). REases are indispensable tools in molecular biology and biotechnology (5–7) and have been studied intensively because of their extraordinary importance for gene analysis and cloning work. In addition, they are important model systems for studying the general question of highly specific protein–nucleic acid interactions (2). REases also serve as examples for investigating structure–function relationships and for understanding the evolution of functionally similar enzymes with dissimilar sequences (3).

Based on subunit composition, cofactor requirements, site specificity and mode of action REases have been classified into four types (8). Enzymes of types I, II and III are parts of restriction–modification (RM) systems, which additionally contain methyltransferases (MTases) adding methyl groups to cytosine or adenine in the host DNA. Type IV REases have no cognate MTases; they recognize and cleave sequences with already modified bases (9) and show only weak specificity (8). RM systems occur ubiquitously among bacteria and archaea (10–12). Their principal biological function is the protection of host DNA against foreign DNA, such as phages and conjugative plasmids (13). Other possible functions are to increase diversity by promoting recombination (13,14) and to act as selfish elements (15,16).

Here we study the recognition sequences of all known type II REases. The main criterion for classifying a restriction enzyme as type II is that it cleaves specifically within or close to its recognition site and does not require ATP hydrolysis. The orthodox type II REase is a homodimer recognizing a palindromic sequence of 4–8 bp. The possible advantage of symmetric recognition sites has already been discussed by the discoverers of restriction enzymes (17). They argued economically that it is ‘much cheaper to specify two identical subunits each capable of recognizing’ the half of the symmetrical sequence than to specify ‘a larger protein capable of recognizing the entire sequence’. This may explain the overwhelming majority of palindromic recognition sequences. However, there are other subtypes too—for instance, type IIA REases that recognize asymmetric sequences (8). Recently, the first example of a type II enzyme (MspI) where a monomer and not a dimer is ‘much cheaper to specify two identical subunits each capable of recognizing’ the half of the symmetrical sequence than to specify ‘a larger protein capable of recognizing the entire sequence’. This may explain the overwhelming majority of palindromic recognition sequences. However, there are other subtypes too—for instance, type IIA REases that recognize asymmetric sequences (8). Recently, the first example of a type II enzyme (MspI) where a monomer and not a dimer binds to a palindromic DNA sequence (18) has been found.

Much has been written about the evolution of REases. When elaborating on this topic Chinen et al. (19) wondered ‘Why are these recognition sequences so diverse?’ Here we show that these sequences are not as diverse as may appear at first sight. Typical patterns can be identified when focusing on purines and pyrimidines. This is apparent from Table 1, which shows the recognition sequences of all restriction enzymes with known three-dimensional structure.

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Watson–Crick base-pairing, without mismatches and without additional ligands were taken into account. The selected PDB entries are 1D8G, 1D8X, 1D23, 1D49, 1EN3, 1EN8, 1ENN, 232D and 295D. The first and last nucleotides in each sequence were omitted from the analysis.

We calculated the average distance between two canonical (22) H-bond donors (and between two acceptors, respectively), each one belonging to one of two adjacent bases. Donor and acceptor pairs must be oriented towards the major or minor groove; pairs with one partner on the major and one partner on the minor groove were omitted. The DNA backbone was not considered for this analysis. Reported distances are averages for the nine selected crystal structures (see Supplementary Table S3). For each dinucleotide base pair we summed all corresponding reciprocal distance values and thus obtained a quantitative measure for H-bond donor and acceptor clusters of each dinucleotide base pair in the major or minor groove (see Supplementary Table S3). The resulting value integrates the number of acceptors/donors and their distance. Simply counting the number of donor and acceptor pairs gives similar results.

Analysis of DNA geometry and flexibility

We analysed four different datasets for the dinucleotide parameters roll, tilt and twist, and three datasets for shift, slide and rise (see Supplementary Table S4). Olson et al. (23) analysed the flexibility in all these six parameters deduced from protein–DNA and pure DNA crystal complexes (yielding two datasets: OlsDNA and OlsProt-DNA). Scipioni et al. (24) deduced the flexibility in roll, tilt and twist from scanning force microscopy images (dataset Scip). Recently (25), all six parameters were calculated from an extensive analysis of structural databases (dataset Per). These authors also found an excellent agreement between database analysis and corresponding molecular dynamics simulations.

RESULTS

Currently, a total of 3726 different REases from 281 bacterial and 26 archaeal genomes are known (REBASE, last update March 3, 2005). The class type II alone comprises 3654 different REases, recognizing 257 different binding sites (the remainder are isoschizomers). Among these are 176 symmetric sequences (mostly recognized by homodimers) and 81 asymmetric sequences. We statistically analysed all type II binding sites and additionally the small datasets of type I, type III and homing endonucleases.

High GC content in DNA binding sites

Our first observation is the significantly enhanced GC content in all type II binding sites: 68% GC and 32% AT. Ambiguous letters (N, R, Y, K and M) were not taken into account (for the complete statistics of base compositions of type II binding sites, see Supplementary Table S1). In contrast, the mean GC content of the host genomes as well as that of the bacteriophages is on average \(\leq 50\%\). The GC content of the binding sites thus deviates significantly from this genome-wide average \((P < 10^{-30})\). We argue that this significantly enhanced GC content reflects biological functionality of the binding sites. Three different facts could play a role in this

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**Table 1.** All type II restriction enzymes with known three-dimensional structure and their cognate DNA recognition sequences [PDB, (20)]

| Enzyme | Source | Recognition sequence | Purine (1)-pyrimidine (0) pattern |
|--------|--------|----------------------|-------------------------------|
| MspI   | Moraxella species | CCGG                  | 0011                          |
| FokI   | Flavobacterium okeanoskete | GGATG               | 11101                         |
| EcoRII | Escherichia coli | CCWGG                | 00W11                         |
| EcoRI  | E.coli    | GAATTCC              | 111000                        |
| BamHI  | Bacillus amyloliquefaciens | GGATCC        | 111000                        |
| HindIII | Haemophilus influenzae | AAGCTT             | 111000                        |
| BglIII | Bacillus globigii | AGATCT              | 111000                        |
| BstYI  | Bacillus steaorthermophilus | RGATCY        | 111000                        |
| EcoRV  | E.coli    | GATATC              | 110100                        |
| Cfr1oI | Citrobacter freundii | RCCGGY            | 100110                        |
| NaeI   | Nocardia aerocolonigenes | GCCGGC          | 100110                        |
| NgoMIV | Neisseria gonorrhoeae | GCCGG               | 100110                        |
| HincII | H.influenzae Rct | GTYRAC              | 100110                        |
| Bse634I| Bacillus species 634 | RCCGGY            | 100110                        |
| MunI   | Mycoplasma species | CAAATG              | 011001                        |
| PvuII  | Proteus vulgaris | CAGCTG              | 011001                        |
| BsoBI  | B.steathermophilus | CYCGRG             | 000111                        |
| EcoO1091| E.coli    | RGGNCCY             | 111000                        |
| BglII  | B.globigii | GCJNNNNNGGC         | 100N110                       |

The corresponding purine (1)-pyrimidine (0) coding shows that 1/00 is a common pattern in all binding sites.

*Recognition sequence representations use the standard abbreviations (21) to represent ambiguity. R = G or A; K = G or T; S = G or C; B = not A (C or G or T); D = not C (A or G or T); Y = C or T; M = A or C; W = A or T; H = not G (A or C or T); V = not T (A or C or G) and N = A or C or G or T.

MATERIALS AND METHODS

All restriction enzyme binding sites were taken from REBASE [last update March 3, 2005 (10)]. Almost all (98%) known REase recognition sequences belong to type II enzymes. We separated the type II binding sites into symmetric and asymmetric sequences, with just 0.96% belonging to the latter class.

The statistical analysis of sequence patterns is based on counting the frequency of all possible substrings up to a length of 4 bp in the symmetric and asymmetric binding sequences (see Supplementary Table S2). In addition to counting substrings of the actual nucleotide sequence, we also counted substrings according to two different binary coding schemes: purine–pyrimidine coding and ketobase–aminobase coding. For the substring analyses of symmetric sequences we consider only the first half of each sequence (the second half is redundant).

Using a binomial distribution, we calculated \(P\)-values that quantify the probability of finding the respective subsequence in a randomized set of binding sites at least as often as in the original binding sites. The \(P\)-values take account of the relative abundance of each letter (A, G, R, N etc.) in the binding sites (see Supplementary Table S1).

Analysis of dinucleotide H-bond donor and acceptor clusters

We selected B-DNA crystal structures from PDB (20) with X-ray diffraction resolution \(\leq 1.5\) Å. Only structures with
In the pur–pyr coding 1 stands for purine (A, G, R) and 0 for pyrimidine (T, C, S), and in the keto-amino coding 1 stands for a ketobase (G, T, K) and 0 for an aminobase (A, C, M).

Enhanced occurrence of RR/YY dinucleotides in DNA binding sites

We separated the type II enzyme recognition sequences into symmetric and asymmetric sequences. In the case of the former we analysed only the first half of the sequence. For these two subsets we counted the occurrence of subsequences up to size 4 and calculated the corresponding P-values (see Materials and Methods and Supplementary Table S2). The most abundant dinucleotides are GG and CC. However, owing to the high GC content (which affects the P-value) the most significant dinucleotide is GA ($P < 10^{-69}$ in the symmetric dataset). Other substrings, such as CTG ($P < 10^{-57}$ in the symmetric dataset) are similarly significant. A much clearer picture is obtained by considering substrings according to the two different binary coding schemes: purine–pyrimidine coding and ketobase–aminobase coding. Table 2 shows that the two dinucleotides RR and YY are the most significant patterns in the large symmetric dataset. In the much smaller asymmetric set, RRR, YYY and YYYY are even more significant, but
RR and YY also stand out. In addition, Table 2 shows that there is no comparably significant ketobase–aminobase pattern. Thus, purine–pyrimidine classification seems to be biologically more important than the ketobase–aminobase categorization. This is also underlined by the fact that among all type II recognition sites the number of Rs and Ys (ambiguous binding sites) is about a factor of 26 higher than the number of Ks and Ms (Supplementary Table S1). REases sometimes allow for some degree of ambiguity, as long as the required purine–pyrimidine pattern is ensured.

The high statistical significance of two and more consecutive purines (or pyrimidines) in type II enzyme binding sites points to biological relevance. We present evidence for three mechanisms that are potentially responsible for the observed enrichment of this pattern.

(i) H-bond donor and acceptor clusters. RR/YY steps provide on average stronger H-bond donor (example in Figure 1) and acceptor clusters than other dinucleotides (see Materials and Methods and Supplementary Table S3). Close proximity of acceptor pairs (or donor pairs) on the DNA allows for the establishment of bifurcated H-bonds, which are stronger than canonical single donor–single acceptor interactions. This feature of RR/YY steps potentially facilitates the recognition and binding of interacting proteins (28). Supplementary Table S3 shows that the average cluster strength of RR/YY steps is higher than that of all other steps. The only (very weak) exception are acceptor clusters in the minor groove, resulting from low strength of the GG/CC step. However, this is counterbalanced by the strong acceptor cluster in the major groove and the donor clusters in the major and minor groove of the GG/CC step. Figure 1 shows an example of a single amino acid (of EcoRI) that potentially interacts with three consecutive purines (GAA) and establishes a bifurcated H-bond.

However, there is growing evidence that specific protein–DNA binding is accomplished not only by specific chemical contacts, but also by suitable geometrical arrangement of the DNA and by its propensity to adopt a deformed conformation facilitating the protein binding (29). The following points (ii and iii) show that both properties are better fulfilled by two adjacent purines (or pyrimidines) than by other dinucleotides.

(ii) Geometrical arrangement. RR/YY steps allow for a special geometrical arrangement of the DNA (see Materials and Methods and Supplementary Table S4). RR/YY steps are characterized by (a) minimal slide values, without exception; (b) strong tilt in the negative direction [dataset Per deviates somewhat, but ‘tilt is a parameter very sensitive to the choice of calculation method’ (30) and, thus, the consistency of the other three datasets seems remarkable]; and (c) a positive roll in all datasets, which implies positive bending towards the major groove (25). The only exception is the AA/TT step in the Scip dataset. However, AA/TT is by far the least significant dinucleotide of all RR/YY steps (Supplementary Table S2).

(iii) Stacking energy. RR/YY steps have a low stacking energy (25) and seem therefore well suited to the often necessary conformational changes during specific protein binding (23,31). Moreover, the stacking energy of all RR/YY steps is anticorrelated with the statistical significance of the RR/YY subsequences (Supplementary Tables S2 and S4). AA/TT has the highest stacking energy and the lowest significance, whereas GA/TC has the lowest stacking energy and the highest significance.

Probably, all three possible reasons for an enhanced frequency of RR/YY steps in type II REase binding sites together play a role in the corresponding specific DNA recognition.

In asymmetric binding sequences longer chains of purines or pyrimidines, such as RRR, YYY and YYYY, are even more significant than RR/YY steps. This could indicate that such substrings are preferred in binding sites. Some dinucleotide parameters, such as stacking energy, more or less add up in longer sequences. On the other hand, a negative correlation between motions at a given base pair step and neighbouring steps was found for most helical coordinates (32).

**Binding sites are underrepresented in host and phage genomes**

The typical features of type II restriction enzyme binding sites, high GC content and overrepresentation of RR/YY steps, could also be linked to the frequency of these sites in the host and/or phage genomes. To address this question we analysed the genome of *E.coli* K12 and the known genomes of its phages (33). All four bases are almost equally abundant in both the *E.coli* genome and the genomes of its phages. Based on this information we can estimate the expected frequency of any given sequence in a randomized genome. Enrichments of sequences are quantified as the ratio of observed versus expected frequency. In addition we calculated weighted ratios, taking into account the number of different enzymes recognizing the same sequence (Supplementary Table S5).

Three findings arise from this analysis: (i) most binding sites are underrepresented in both the host and the phage genomes (possible explanations are that phages try to escape REases and that hosts minimize the methylation effort); (ii) under-overrepresentation in host and phage genomes is correlated; and (iii) under-overrepresentation is correlated with GC content and RR/YY frequency (most underrepresented sequences contain only GC and always contain RR/YY steps). This
correlation again underlines the biological importance of these two features.

**DISCUSSION**

We presented a statistical analysis of all known DNA recognition sites of type II restriction enzymes. This collection comprises by far the largest group of reliably known specific protein binding sites on DNA. There is hardly any sequence similarity among restriction enzymes (34). REases often use uncommon DNA binding motifs (35), but sometimes also typical structures already known from transcription factors, such as FokI and Nael, which both use a helix–turn–helix motif. The typical features of type II REase binding sites such as high GC content and many RR/YY steps may also be relevant for other DNA recognition sequences. We have also analysed all known binding sites of type I and type III restriction enzymes and of homing endonucleases (Supplementary Tables S6–S8). However, we found no statistically significant motifs, which is probably due to the small number of sequences of these types. Homing endonucleases are known to bind less specifically (10,36). This lack of specificity could be another explanation for the lack of statistically significant patterns among this class of binding sites. Table 3 shows examples of other DNA binding proteins along with their recognition sequences. Nearly all of them contain RR/YY steps. The average GC content of these sequences is 54%.

We presented three different possible explanations for the amplified occurrence of two neighboured purines (or pyrimidines) in the recognition sites. One argument is that these give stronger H-bond donor and acceptor clusters than any other adjacent base pair and therefore facilitate hydrogen bonds to amino acids. For instance, EcoRV (binding GATATC) establishes multiple contacts to the first 2 bp and the last 2 bp, but none to the middle 2 bp (60).

Evolutionary relatedness of REases recognizing similar sequences would be a completely different explanation for our observed patterns. Although only a few REase crystal structures have been solved so far, it became clear from additional bioinformatics studies that REases belong to at least four unrelated and structurally distinct superfamilies: PD-(D/E)XK, PLD, HNH and GIY-YIG (34). The largest one [PD-(D/E)XK] comprises the two major classes α (EcoRI-like) and β (EcoRV-like) (2). Enzymes belonging to the same superfamily sometimes also have similar recognition sequences. For instance, Eco29kI, NgoMIII and MraI, which are related to the GIY-YIG superfamily, all bind to CCGCGG (61). HpyI (CATG), NlaIII (CATG), SphI (GCATGC), NspHI (RCATGY), NspI (RCATGY), MboII (GAAGA) and KpnI (GGTACC) belong to the HNH superfamily (62), and SsoII (CCNGG), EcoRII (CCWGG), NgoMIV (GCCGGC), PspGI (CCWGG) and Cfr10I (RCCGGY) to the EcoRI branch (63). It has already been argued that these enzymes diverged early in evolution, presumably from a type IIP enzyme that recognized...

**Table 3. Examples of gene regulatory proteins that recognize specific short DNA sequences**

| DNA binding protein | Recognition sequence (or consensus motif) | Purine (1)-pyrimidine (0) pattern | References |
|---------------------|------------------------------------------|----------------------------------|------------|
| p53                 | RRRCW,GYYYRRRCW,GYYY                     | 1110W210001110W11000            | (38)       |
| MADS box            | CCW,GG                                   | 00W1,11                         | (39)       |
| ERSE                | CCAATN6CCACG                             | 00110N0,0101                    | (40)       |
| Ski oncoprotein     | GTCTAGAC                                 | 10001110                        | (41)       |
| GAL4                | CGGN,TTNCGG                              | 011N06001                        | (42)       |
| GAL4 in vitro       | WGGN10–1CCGG                             | W11N010–1000                     | (42)       |
| nix-2.5             | CWTTAAATNN                               | 0W0011000                       | (43)       |
| Bicoid              | TCTAATCC                                 | 0001100000                      | (44)       |
| AP-2                | GCCCCAGGC                                | 100001110                       | (45)       |
| Stat5-RE            | TTCN,GAA                                 | 000N1,11                        | (46)       |
| GRE                 | AGAACAN,TGTCTTCT                          | 111101N0100000                  | (46)       |
| SRF                 | CCW2,AW,GG                               | 00W2,1W11                       | (47)       |
| MCM1                | CYYW,N1GG                                | 000W2,1N11                      | (47)       |
| NFXB                | GGACTTCC                                 | 111100000                      | (48)       |
| pur repressor       | ANGCCAACNTNCT                             | 11N0101N0000N0N0N0              | (49)       |
| Y71                 | GGCCATCTTG                               | 11001000000                    | (50)       |
| NF-I/CTF-1          | TGGN6GCCA                                | 011N610011                      | (51)       |
| PPAR                | AGGAAACTTGA                               | 11111100011                     | (52)       |
| NFAT                | ATTGGAAA                                 | 10011111                       | (53)       |
| CREA                | GGCGAGACCCAG                              | 10111100010011                  | (54)       |
| C/EBP               | CCAAT                                    | 001101                          | (55)       |
| PaC                 | GCCARG                                   | 1001111                        | (56)       |
| TTK finger1         | GAT                                      | 110                            | (57)       |
| TTK finger2         | AGG                                      | 111                            | (57)       |
| Zif finger1         | GCG                                      | 101                            | (57)       |
| Zif finger2         | TGG                                      | 011                            | (57)       |
| GLI finger4         | TTGGG                                    | 001111                         | (57)       |
| GLI finger5         | GACC                                     | 1100                           | (57)       |

**E.coli sigma factors**

(binding in −35 region)

| σ70 (primary)       | CTTGA                                    | 000111                         | (58–60)    |
| σ32 (heat shock)    | CTTGAA                                   | 000111                         |            |
| σ60 (nitr. reg. gene) | CTGONA                              | 0011N1                         |            |
| σ54 (nit. ox. stress) | TTGG,CACG                              | 00111011                      |            |
| σ28 (exter. stress) | CTAAA                                    | 001111                         |            |
CCxGG or xCCGGx (63). We are not aware of any systematic study of recognition sequence similarity versus membership in superfamilies. However, it is conceivable that sequence similarity (or the corresponding purine–pyrimidine pattern) is evolutionarily conserved. Some positive correlation between amino acid similarity and recognition sequence similarity of restriction enzymes has already been found (64). However, REases are extremely divergent and mostly structurally and evolutionarily unclassified (34). Even related enzymes binding to similar DNA sequences may differ much in the details of protein–DNA interaction. Comparing the cocrystal structures of two related enzyme pairs BglII (AGATCT) versus EcoRI (GAATTTC), which both differ in only the outer base of the binding site. For the first pair they found ‘surprising diversity’ in how the common base pairs are recognized, whereas the enzymes of the second pair recognize their common inner and middle base pairs in a nearly identical manner.

The problem of recognition and binding of a protein to its specific DNA sequence is far from being solved. Heitman and Model (35) substituted amino acids in the binding domain of EcoRI such that some of the original 12 hydrogen bonds contacting the base pairs of the recognition sequence could not be established by the mutant. This change did not affect the binding specificity of EcoRI, but only its enzymatic activity. It was concluded that the hydrogen bonds revealed by the crystal structure are insufficient to fully account for substrate recognition, and additional amino acids must contact the DNA to help discern the substrate (35). The authors argued that protein–DNA interactions can be influenced by sequence-dependent variation of the structure of the DNA backbone [originally suggested by Dickerson (67)], and that the EcoRI enzyme could recognize its cognate sequence because it adopts its unusual bound conformation more readily than other DNA sequences. It was concluded that even with a detailed cocrystal structure it is exceedingly difficult to determine which interactions contribute to sequence-specific DNA recognition (35). Moreover, it has been found that protein binding to DNA is modulated by sequence context outside the recognition site (68) and that different endonucleases have different context preferences (69).

Our work suggests that sometimes only the purine–pyrimidine pattern matters for recognition by a certain biomolecule. Note that R and Y are most frequent among the ambiguous letters in restriction enzyme binding sites. In such cases the exact base would be irrelevant as long as it is a purine (or pyrimidine). Several such examples are already known. For instance, during translation the third base of the codon is nearly always analysed in this binary manner (in the yeast mitochondrial code this is always the case) (70). Another example is the sequential contact model for EcoRI, proposing that during the transition from DNA binding to DNA scission, the contacts to the pyrimidines could either precede or follow the purine contacts observed in the crystal structure (35). It is known that a change in just 1 bp of the cognate site can reduce the ratio $k_{cat}/K_m$ for DNA cleavage by a factor of $>10^6$ (71). Thus, a transition exchange might generally have a less dramatic effect than a transversion exchange. Such a smaller effect of a transition exchange could also be observed in corresponding pausing experiments (72), which might be important for protein engineering.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at NAR Online.

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