Definition of the Sequence Requirements for Binding of the EBNA-1 Protein to Its Palindromic Target Sites in Epstein-Barr Virus DNA

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Interaction between the *trans*-acting DNA-binding protein EBNA-1 and *cis*-acting sequences in the ori-P region of Epstein-Barr virus DNA is required for maintenance of the viral plasmid state in latently infected B cells and is involved in the regulation of transcription during latency. In the Epstein-Barr virus genome, a total of 26 EBNA-1-binding sites occur within three clustered loci referred to as the family of repeats and dyad symmetry locus of ori-P and the separate *Bam*HI-Q locus. Incubation of a bacterially expressed carboxy-terminal domain of EBNA-1 (28,000-molecular-weight EBNA-1 [28K EBNA-1]) with synthetic monomer and dimer consensus binding sites gave characteristic DNA-protein complexes in a mobility retardation assay. A similar approach with the naturally occurring Q locus confirmed that it contains two distinct but low-affinity binding sites. We then examined the precise sequence requirements for EBNA-1 binding, using a set of 30-base-pair oligonucleotides designed to contain symmetric point mutations within both halves of the palindromic target site. Analysis of all possible single substitutions between positions 1 and 10 in the consensus half-palindrome sequence revealed that positions 9 and 10 did not contribute to EBNA-1 binding and that considerable flexibility could be tolerated at positions 1 and 2. Positions 3 through 8 of the recognition site had the most stringent requirements, with transversions at these positions either reducing or eliminating binding. The relative spacing of the halves of the palindrome was also critical, since the addition or removal of 2 base pairs at the center of the sequence abolished binding. Similar results were obtained when a partially purified preparation of intact Raji EBNA-1 was substituted for the 28K EBNA-1, and the results were further supported by methylation interference studies which indicated contact points between EBNA-1 and the guanine residues at positions -8, -7, and +3 of the binding site. The three naturally occurring EBNA-1-binding loci have previously been shown to differ in their relative affinities for EBNA-1. The present study indicates that the sequence variations occurring within the family of repeats would not affect binding affinity, whereas certain base substitutions within the Q and dyad symmetry sites would be predicted to contribute to the observed lower affinities of these sites. An apparent K_d of 1.5×10^{-11} M for binding of 28K EBNA-1 to a consensus recognition site was calculated from Scatchard analysis.

Epstein-Barr virus (EBV) infection of B cells leads to the establishment of viral latency during which the EBV genome is maintained extrachromosomally as a multicopy, circular plasmid (9, 12). The viral nuclear antigen EBNA-1 has a central role in the maintenance of latency. Plasmids containing a DNA element from the EBV genome (ori-P) are maintained in an unintegrated state if EBNA-1 is concomitantly expressed in the cell, suggesting that EBNA-1 is the only viral protein required for plasmid maintenance (24, 25). ori-P, the minimal *cis*-acting sequence necessary for maintenance of the DNA extrachromosomally, consists of two essential domains, the family of repeats (region I), which consists of 20 tandem copies of a 30-base-pair (bp) repeat unit, and the dyad symmetry region (region II), which contains four copies of the same repeat. The family of repeats exhibit EBNA-1-dependent enhancer activity (14, 22, 23), and evidence has been presented that the dyad symmetry locus is the site of initiation of viral DNA replication (6, 23). These two loci are separated by approximately 1,000 bp, and the relative orientation of the loci and the spacing between them can be altered without affecting ori-P function (5, 15).

Reisman and Sugden (14) demonstrated that, in recombi-

nant plasmid constructions, the family of repeats act as an EBNA-1-dependent enhancer. Although these repeats lie approximately 3 and 6 kilobases, respectively, distant from the *Bam*HI-C and *Bam*HI-W latency promoters that control transcription of the EBNA family of genes (2, 3, 17, 20), the possibility was raised that EBNA-1 might positively regulate expression of the EBNA latency proteins. Experiments by Sugden and Warren (22) have now demonstrated directly that EBNA-1 can transactivate the *Bam*HI-C latency promoter via the ori-P enhancer region. Thus the EBNA-1 protein is functionally pleiotropic, playing a regulatory role in both viral episomal replication and transcription during latency.

EBNA-1 function proved to be mediated via sequence-specific DNA binding to the domains of ori-P. Rawlins et al. (13) used DNase I protection studies to demonstrate that the sequences within the family of repeats represent 20 tandem binding sites for EBNA-1 while the dyad symmetry region contains 4 overlapping binding sites. An additional binding site locus was also identified in a separate region of the genome within *Bam*HI-Q. The functional role of the Q locus has yet to be determined. The affinity of EBNA-1 for the three binding loci differs significantly, with the family of repeats having the greatest binding affinity, the dyad symmetry region having an intermediate affinity, and the Q locus

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having the weakest affinity for EBNA-1 (7). These differences in affinity may provide part of the mechanism through which EBNA-1 modulates both replication and latency transcription and hence we were interested in determining their basis. Differences in affinity could theoretically arise in a number of ways, for example, through cooperativity between binding sites or as a result of DNA sequence differences within the individual binding sites. Previous studies had found no significant role for cooperativity between binding sites within the family of repeats (7, 10), and therefore we turned our attention to the contribution of the naturally occurring sequence variations within the EBNA-1-binding loci. We prepared synthetic oligonucleotide binding sites containing all possible combinations of symmetric single base changes, within positions -10 to -1 (+1 to +10) of the consensus site, along with some multiple changes, and measured their properties of binding to a bacterially expressed 28,000-molecular-weight EBNA-1 (28K EBNA-1) protein (11) by using gel retardation assays. These oligonucleotide mutational studies yielded experimental evidence that primary sequence variations provide a basis for the observed relative affinities of the three EBNA-1-binding loci in the EBV genome.

MATERIALS AND METHODS

Preparation of EBNA-1. The EBNA-1 fusion protein was prepared by a modification of previously described protocols (11, 13). *Escherichia coli* K-12 DH1 containing pNAK28 was grown overnight at 30°C. The overnight cultures were diluted 100-fold into 5 ml of broth and grown at 30°C for 3 h. Protein expression was induced by transferring the cultures to a 42°C water bath for 1.5 h. The cells were pelleted by centrifugation and washed in 5 ml of 50 mM Tris hydrochloride (pH 7.5). The repelleted cells were transferred to a 1.5-ml Microfuge tube and suspended in 100 µl of solution containing 50 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and then frozen at -80°C and thawed with the addition of an equal volume of 50 mM Tris hydrochloride (pH 7.5)-10 mM EDTA-1 mM phenylmethylsulfonyl fluoride-0.5 mg of lysozyme per ml-0.1% Nonidet P-40. The solution was mixed with 80 µl of 5 M NaCl, and the cell debris was removed by centrifugation for 10 min at 10,000 × *g* in a Microfuge. Partially purified 28K EBNA-1 was obtained by heparin-agarose chromatography (13). Intact 80K EBNA-1 protein from Raji cells was purified as previously described (13).

Preparation of oligonucleotide- and DNA fragment-binding substrates. Oligonucleotides were synthesized on a 380B synthesizer (Applied Biosystems) and high-pressure liquid chromatography purified by the Johns Hopkins Nucleotide Synthesis Service. Concentrations of the oligonucleotides were determined by A_{260} and confirmed by gel electrophoresis on a 10% polyacrylamide gel and ethidium bromide staining. Symmetric consensus and mutant oligonucleotide binding sites were prepared from 19-mer synthetic single-stranded oligonucleotides which could self-anneal at their palindromic 3' ends and thus serve as primers for a Klenow fill-in reaction, generating double-stranded 30-mers (Fig. 1). Oligonucleotides (0.1 µg) were end labeled with [γ - 32 P]ATP (10 µCi) in a 10-µl reaction volume and washed with 2 ml of TE (10 mM Tris hydrochloride, 0.1 mM disodium EDTA, pH 7.4) by using a Centricon-10 filtration device (Amicon Corp., Danvers, Mass.). The retentate was dried in a vacuum concentrator, suspended in 10 µl of 3× buffer (30 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 30 mM MgCl₂), and

FIG. 1. Mode of synthesis of the symmetric double-stranded oligonucleotides used in the EBNA-1-binding assays. Single-stranded oligonucleotide primers (19-mers) were self-annealed to form the structures shown in sequence 1. Incubation of this intermediate with the Klenow fragment of DNA polymerase I resulted in formation of a 30-mer double-stranded substrate, as indicated in sequence 2.

allowed to anneal for 1 h at 37°C. Deoxynucleoside triphosphates were then added to a final concentration of 250 µM, the reaction mixture was diluted to a final volume of 30 µl, and 5 U of Klenow fragment of *E. coli* DNA polymerase I was added to the mixture. After a 1-h incubation the mixture was washed with 2 ml of STE (150 mM NaCl, 10 mM Tris hydrochloride, 0.1 mM disodium EDTA, pH 7.4) by using a Centricon-20 filtration device, and the retentate was diluted to 100 µl in STE.

Specific DNA fragment probes were prepared from plasmids pGH66, pGH65, pDH145, and pDH144, all of which have been described previously (7). Plasmids pGH66 and pGH65, carrying one and two copies, respectively, of a consensus 30-mer binding site from the family of repeats, were digested with *Eco*RI and *Xba*I to generate 90- and 120-bp DNA fragments which were end labeled with Klenow polymerase and [γ - 32 P]ATP. Plasmid pDH145 was digested with *Hind*III and *Bam*HI to generate a 310-bp fragment carrying the Q locus and similarly end labeled. Plasmid pDH144 was cleaved with *Eco*RI, end labeled, and then digested with *Eco*RV to generate a 191-bp fragment containing the intact dyad symmetry region. The individual DNA fragments were isolated after separation on a 5% polyacrylamide gel and purified by phenol-chloroform extraction and ethanol precipitation.

Mobility retardation assays and Scatchard analysis. EBNA-1 extract (0.1 µl) was incubated with probe DNA (1 fmol) for 30 min at room temperature in a 25-µl total volume of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.5])-1 mM dithiothreitol-5 mM MgCl₂-1 mg of bovine serum albumin per ml-100 mM KCl-0.1% Nonidet P-40-1 µg of poly(dI-dC) (Pharmacia, Inc., Piscataway, N.J.). The reaction mixtures were loaded onto a 10% polyacrylamide gel in 1/2× TBE (20× = 1 M Tris hydrochloride, 1 M boric acid, 20 mM EDTA)-0.05% Nonidet P-40 and electrophoresed in 1/2× TBE at 10 V/cm at room temperature for 1.5 h. The gels were dried and autoradiographed, and shifted and unshifted bands were excised and counted in scintillant (Betafluor).

The substrate for the Scatchard analysis was formed by annealing the 30-mer consensus oligonucleotides used in the construction of pGH65 and pGH66 (7) (5'-GATCCAGATTAGGATAGCATATGCTACCCA-3' and 5'-GATCTGGGTCATATGCTATCCTAATCTG-3') and end labeling with [γ - 32 P]ATP and polynucleotide kinase. The binding reaction mixture consisted of 32 P-labeled double-stranded oligonucleotide binding site (0.1 fmol), 2 µl of 28K EBNA-1 extract, and increasing amounts of the unlabeled consensus oligonucleotide (0, 0.03, 0.06, 0.3, 0.6, or 3 fmol). The total bound oligonucleotide ([PD_s]) was estimated as the product of the fraction of 32 P-labeled oligonucleotide in the shifted position and the total concentration of oligonucleotide (labeled and

unlabeled) in the reaction mixture. The free oligonucleotide ($[D_s]$) was estimated as the product of the fraction of ^{32}P -labeled oligonucleotide in the unshifted position and the total concentration of oligonucleotide in the reaction. The results were plotted according to

$$[\text{PD}_s] = [P^0]K_{\text{app}}[D_s] \quad (1)$$

$$\text{and } [\text{PD}_s]/[D_s] = K_{\text{app}}[P^0] \quad (2)$$

where $[P^0]$ is the molar concentration of specific DNA-binding species present at the start of the experiment and is estimated from the x intercept of the Scatchard plot and K_{app} is the apparent equilibrium association constant and is estimated from the negative slope of the Scatchard plot (1, 4). The apparent equilibrium dissociation constant K_d is $1/K_{\text{app}}$.

Methylation interference. A 191-bp *EcoRI-EcoRV* DNA fragment containing the intact dyad symmetry region from plasmid pDH144 formed the probe for the methylation interference assay. A portion of this probe, which was end labeled at the *EcoRI* site, was used to generate a control DNA sequence ladder, and the remainder was treated with dimethyl sulfate (19, 21). A sample of DNA (10 μl) was diluted into 190 μl of 50 mM sodium cacodylate (pH 7.1)–1 mM EDTA and reacted with 1 μl of dimethyl sulfate (freshly diluted 1:40 in ethanol) for 15 min at 37°C. The reaction was terminated by the addition of 50 μl of 1.5 M sodium acetate containing tRNA (100 $\mu\text{g/ml}$), and the DNA was ethanol precipitated. The binding reaction mixture contained sufficient EBNA-1 to shift 50% of the modified probe into the fully complexed form. Bound and unbound DNA were separated on a 5% acrylamide gel, isolated, cleaved with piperidine, and electrophoresed through a 6% denaturing gel. The gel was dried and exposed to X-ray film overnight.

RESULTS

BamHI-Q locus contains two functional binding sites. The studies of Rawlins et al. (13) demonstrated by filter-binding assays that a 3.9-kilobase *BamHI* subfragment from the EBV (MABA) cosmid cMB-14 contained a third EBNA-1-binding locus, one that maps within the *BamHI* Q fragment and therefore lies outside the *ori-P* region required for maintenance of the plasmid state. Examination of the DNA sequence of *BamHI*-Q revealed two potential EBNA-1-binding sites, with their centers 21 bp apart. However, the sequences of both of these potential sites are substantially diverged from the family of repeats consensus sequence, and it was not known whether only one or both of these sites were capable of binding EBNA-1 protein. As a first step toward examining this question, a 90-bp *EcoRI-XbaI* DNA fragment from pGH66 and a 120-bp *EcoRI-XbaI* DNA fragment from pGH65, which contain one and two copies, respectively, of a consensus 30-mer EBNA-1-binding site, were isolated. The fragments were end labeled and incubated with a preparation of bacterially expressed 28K EBNA-1 that had been partially purified by passage over a heparin-agarose column. Specific binding activity was assayed by mobility retardation (19). The titration experiment whose results are shown in Fig. 2a and b revealed that the 90-bp fragment containing a monomer insert produced just a single shifted band, while the 120-bp fragment could be shifted into first one and then a second specific slower-migrating complex. A nearly identical 60-bp DNA fragment without the inserted oligonucleotide consensus site failed to bind (results not shown). We interpret this result to indicate that the two shifted forms obtained with the 120-bp fragment

represent occupancy of first one and then both copies of the consensus sequence and that the gel retardation assay can be utilized to determine the number of EBNA-1-binding sites within a given DNA fragment.

To examine the structure of the *BamHI*-Q locus a mobility retardation assay was performed, using a 310-bp *HindIII-BamHI* fragment from plasmid pDH145 that encompassed both potential EBNA-1-binding sites and contained nucleotides 62249 to 62553 from the B95-8 genome. The 310-bp fragment produced two shifted bands (Fig. 2c), indicating that both of the predicted sequences within *BamHI*-Q in fact represent functional binding sites. EBNA-1 binding was quantitated from densitometer scans of the autoradiographs (Fig. 3). The data confirms previous observations (7) that the Q locus has a lower affinity for EBNA-1 than a consensus binding site from the family of repeats. In these experiments, 14 times more protein was required to achieve 50% occupancy of the first Q-binding site compared with the first consensus binding site. The data also suggest that the two Q-binding sites differ in affinity by at least a factor of 2. If both sites were of equal affinity, then the amount of protein required to give 50% occupancy of two sites should be twice that required for a single site. In the case of the consensus dimer, the experimentally determined value is 1.6, whereas for the Q locus 3.5 times more protein was required, a twofold difference.

Direct mobility shift assays with uncloned mutant oligonucleotides. To carry out a detailed evaluation of the base sequence requirements for EBNA-1 recognition of the binding site, we developed a simplified assay for binding, involving synthesis of a single partially self-complementary 19-bp oligonucleotide, which after annealing could be extended into a 30-bp double-stranded molecule by using Klenow DNA polymerase (Fig. 1). Note that this protocol results in a perfectly palindromic binding site and that all mutations which are introduced will be introduced symmetrically into both halves of the binding site. The uncloned forms of these oligonucleotides were assayed directly by the mobility retardation procedure, using crude pNAK28 bacterial extracts.

In an initial set of experiments, we tested the 30-bp double-stranded forms of a parent consensus oligonucleotide (oligonucleotide no. 20) (Table 1) plus three variants with symmetric multiple point mutations at positions 7 to 10 (no. 21), 4 to 6 (no. 22), and 1 to 3 (no. 23) relative to the center of the palindromic binding site. The parent-duplicated consensus half-palindromic binding site (half-site) containing the sequence 5'-TGGATAGCAT-3' at positions -10 to -1 on the top strand and at +1 to +10 on the bottom strand (no. 20) gave a discrete shifted complex in 10% polyacrylamide gels, but none of the mutated oligonucleotides demonstrated any binding (Fig. 4a). Similarly, a 32-bp oligonucleotide site with a 2-bp AT insert between positions -1 and +1 at the center of the palindrome (no. 30) and another 28 bp in length with the A and T bases at positions -1 and +1 deleted (no. 69; results not shown) failed to bind. The sensitivity of these assays was usually such that a minimally detectable shifted band in the autoradiograph represented between 2 and 3% of the radioactivity present in the shifted band from the control consensus oligonucleotide.

The results described above indicated that the spacing between the two halves of the palindrome and the base sequences in both the central and outside positions within the palindrome were all critical for EBNA-1 binding. Therefore, for a more precise evaluation we prepared a set of 10 single-base transversion mutations, one at each position from -10 to -1 (+1 to +10). The binding results with 28K

FIG. 2. Mobility retardation assays showing binding of 28K EBNA-1 to monomer and dimer consensus binding sites and to the Q locus. Increasing amounts of 28K EBNA-1 were incubated with a 90-bp *EcoRI-XbaI* DNA fragment containing one copy of a consensus binding site from the family of repeats (a), a 120-bp *EcoRI-XbaI* fragment containing two copies of the consensus binding site (b), and a 310-bp *HindIII-BamHI* fragment that encompassed both sites within the Q locus (c). F, Free probe; B, bound probe. One unit of EBNA-1 equals the amount of protein which, when incubated with 1 fmol of the 90-bp fragment from pGH66, results in 50% of the probe migrating in the bound form.

EBNA-1 extracts for each of these 30-bp oligonucleotides are also shown in Fig. 4a. Conditions were chosen under which approximately 80% of the parent consensus oligonucleotide was migrating at the shifted position. For quantitation, the fraction of radioactivity present in the shifted band for each mutant oligonucleotide was expressed as a percentage of the consensus fraction (Fig. 4c). The results implied that positions -7, -6, -5, -4, and -3 were critical for binding but that positions -10, -9, -2, and -1 were not. The change at position -8 had an intermediate effect (30% binding).

Consequently, we prepared several putative minimal palindromic EBNA-1-binding sites based on the half-site consensus oligonucleotide NNNATAGCNN but having substitutions of ACC in place of TGG at positions -10 to -8 or CG

in place of AT at positions -2 and -1. However, oligonucleotides with only the ACC substitution (oligonucleotide no. 66), with only the -2 and -1 CG substitutions (no. 67), or with both of these substitutions (no. 68) all either failed to bind or gave extremely weak binding activity, whereas an AC for TG substitution at positions -10 and -9 (no. 86) bound normally (Table 1). Since these results were not consistent with our initial interpretation of the data from the transversion series, we concluded that a more complex situation must prevail.

Comparison of the binding of bacterial 28K EBNA-1 and mammalian 80K EBNA-1 proteins. Before proceeding further with this evaluation, it seemed important to demonstrate that the data obtained for the bacterially expressed EBNA-1 fusion protein accurately reflected the target specificity displayed by the intact, authentic EBNA-1 protein. Consequently, a heparin-agarose-purified fraction of 80K EBNA-1 extracted from Raji cells (7) was used in similar direct shift-up assays with the transversion mutant oligonucleotide series. Although the binding activity present was relatively low and we were unable to find conditions under which the shifted complexes with the intact mammalian protein entered the gels, we were able to quantitate the binding on the basis of the proportion of radioactivity lodged at the top of the gel (Fig. 4b). The overall results with the multiple point mutations, the 2-bp insertion, and point-mutant oligonucleotides proved to be very similar for the two protein preparations. A quantitative comparison of the binding efficiency in parallel sets of binding studies with the transversion mutants (Fig. 4c) revealed that although the G to T change at position -8 resulted in 30% of wild-type binding with the bacterial 28K EBNA-1, this change had a more dramatic effect with the Raji lymphocyte-derived protein (5% binding). Experiments described in the following section confirmed the importance of the -8 position, where a G to C change (oligonucleotide no. 196, Table 1) also resulted in minimal binding (5%) with the 28K EBNA-1 protein. The Raji EBNA-1 demonstrated somewhat greater stringency at positions -2 and -1 than the bacterial preparation, with transversions at these positions reducing binding to 42 and 35% of wild-type levels. This result proved typical of the more subtle influence of the -2 and -1 positions.

Binding properties of a complete set of half-site point mutants. Although there are several variations from the standard consensus sequence among them, the first 20 adjacent copies of the 30-bp repeats within the ori-P region I of EBV (B95-8) DNA have approximately equal affinity for 28K EBNA-1, as judged by our earlier footprinting analysis (13). However, a directly adjacent slightly diverged repeat of this sequence (site "21") failed to bind in those studies. In contrast to region I, the six additional binding sites represented in the dyad symmetry and Q loci are more diverged from the standard consensus sequence and have either intermediate or low-level affinities for the EBNA-1 protein (7). In an attempt to assess the precise sequence requirements for EBNA-1 binding, we prepared oligonucleotides containing all possible combinations of symmetric single base changes within positions -10 to -1 of the consensus sequence and measured their binding properties for comparison with the naturally occurring variations. The relative percent binding of each oligonucleotide is listed in Table 1 and represents an average of between three and eight separate determinations. In two instances, when the consensus guanine at position -8 was substituted by cytidine and when the consensus cytidine at position -3 was substituted by guanine, an extended region of self-homology was created

FIG. 3. Quantitative comparison of 28K EBNA-1 binding to the consensus binding sites versus the Q locus. The data were obtained from densitometer tracings of the mobility retardation assays shown in Fig. 2.

and the Klenow fill-in reaction yielded a smaller, 24-bp product instead of the desired 30-mer. This problem was circumvented by the superimposition of a guanine for adenine at position -7 to create oligonucleotides 196 and 197. At position -7, guanine occurs as frequently as adenine in the naturally occurring binding sites, and data from our mutated oligonucleotides suggested no difference in binding activity when guanine was substituted for adenine at this position in the consensus (oligonucleotide no. 70, Table 1). Note that in contrast to undetectable binding (<2%) with some of the multiple mutants described above, all single point mutants that were rated marginal nevertheless showed detectable binding, with the exception of no. 101. The naturally occurring variations within the family of repeats, dyad symmetry, and Q loci are shown in Table 2, along with the oligonucleotides containing those particular mutations.

The results for all single-nucleotide variations assayed within the standard consensus half-site context of the symmetric oligonucleotides are also summarized diagrammatically in Fig. 5. Binding activity of between 70 and 150% relative to that of the control consensus oligonucleotide was considered strong, whereas a binding level of between 20 and 40% was considered weak. Binding between 2 and 8% of normal was considered marginal. The diagram also summarizes the consensus sequence pattern observed among all naturally occurring EBNA-1 half-sites in a similar way. Bases that are nearly always present and variations that occur outside the family of repeats are indicated. Substitutions that have not been found in any naturally occurring sites are left blank.

Despite the much narrower implications from the initial transversion series, the complete analysis of all single-point variations between positions -8 and +8 showed rather complicated base sequence influences and only positions 10 and 9 turned out to be completely unimportant. The most significant points to draw from the data within the central 16 bp are as follows. (i) Position 5 has the only totally invariant base, with both sides of all naturally occurring binding sites being A. The results with bacterial EBNA-1 and the synthetic oligonucleotides were in substantial agreement, with C and G being forbidden variations, although a substitution with T at positions -5 and +5 in the consensus did give weak binding. (ii) Among the naturally occurring palindro-

mic target sequences, only position 7 had two bases appearing at approximately equal frequencies. In most cases, G occurred at -7 in one half-site and A occurred at +7 in the other half-site, although several examples with two As and others with two Gs also occurred. Similarly, in the oligonucleotide studies either A or G was acceptable at positions -7 and +7 but C and T were not. (iii) At most other positions (i.e., -6, -4, -3, and -2), two alternative bases gave strong binding and at least one base was not permitted. Furthermore, at position -1, three alternatives (A, C, and G) were apparently acceptable (although see below). (iv) None of the naturally occurring minor variations found within the family of repeats (T at position -3 and C at -2) had deleterious effects within the oligonucleotides. This was also the case for two of the four substitutions in the dyad symmetry region (A at position -8 and A at -4) and for three of the five substitutions found in the Q locus (C at position -1, T at -3, and A at -4). (v) The only naturally occurring variations that were not permissible in the duplicated form in the oligonucleotides were the substitutions of G at +6 which occurs in one of the Q sites, the Gs at -2 and +2 present in the other Q site, and T at -4 in site 21 of the family of repeats. (vi) Only one substitution that has not been found naturally (G at position -1) had no inhibitory effect on binding.

Effect of multiple base changes. Within the central 4 bp of the palindrome there is only one minor substitution (C at position -2 occurring four times) within the total of 50 half-sites represented by the family of repeats and the dyad symmetry region (including site 21), whereas four additional substitutions at -2 or -1 occur within the four half-sites in the Q locus. Furthermore, it seemed curious that either an A to C substitution at -2 and +2 (oligonucleotide no. 39) or a T to G substitution at -1 and +1 (no. 40) was perfectly acceptable within the oligonucleotides but that the double substitution of CG for AT (no. 67) reduced binding to only a weak level. Therefore, several other double substitutions at these positions were also tested, and the results were rather surprising (Table 1). Although the single change of A to G at -2 (no. 99) abolished binding, the addition of a T to C change at -1 (no. 104) appeared to compensate to some extent by restoring weak binding activity. Even more dramatically, although both single changes of A to T at -2 (no. 100) and T to A at -1 (no. 101) abolished binding (less than

TABLE 1. Summary of relative binding of bacterial 28K EBNA-1 to mutant oligonucleotides

Mutation type and oligonucleotide no.	Half-palindrome position no. ^a											% Relative binding affinity (no. of assays)
	±10	±9	±8	±7	±6	±5	±4	±3	±2	±1		
Single point												
20	T	G	G	A	T	A	G	C	A	T		100
31	<u>G</u>	G	G	A	T	A	G	C	A	T		108 (3)
137	<u>C</u>	G	G	A	T	A	G	C	A	T		154 (8)
32	T	<u>T</u>	G	A	T	A	G	C	A	T		92 (6)
136	T	<u>A</u>	G	A	T	A	G	C	A	T		146 (8)
135	T	G	<u>A</u>	A	T	A	G	C	A	T		108 (8)
70	T	G	G	<u>G</u>	T	A	G	C	A	T		109 (4)
75	T	G	G	A	<u>C</u>	A	G	C	A	T		93 (3)
73	T	G	G	A	T	A	<u>A</u>	C	A	T		79 (4)
74	T	G	G	A	T	A	G	<u>T</u>	A	T		89 (4)
197 ^b	T	G	G	G	T	A	G	<u>G</u>	A	T		70 (3)
39	T	G	G	A	T	A	G	C	<u>C</u>	T		83 (5)
102	T	G	G	A	T	A	G	C	A	<u>C</u>		84 (4)
40	T	G	G	A	T	A	G	C	A	<u>G</u>		104 (3)
33	T	G	<u>T</u>	A	T	A	G	C	A	T		30 (6)
71	T	G	G	A	<u>A</u>	A	G	C	A	T		29 (4)
96	T	G	G	A	T	<u>T</u>	G	C	A	T		40 (3)
196 ^b	T	G	<u>C</u>	G	T	A	G	C	A	T		5.0 (3)
34	T	G	G	<u>C</u>	T	A	G	C	A	T		3.4 (5)
95	T	G	G	<u>T</u>	T	A	G	C	A	T		4.5 (4)
35	T	G	G	A	<u>G</u>	A	G	C	A	T		2.5 (5)
36	T	G	G	A	T	<u>C</u>	G	C	A	T		3.2 (5)
72	T	G	G	A	T	<u>G</u>	G	C	A	T		2.5 (4)
37	T	G	G	A	T	A	<u>T</u>	C	A	T		3.4 (7)
97	T	G	G	A	T	A	<u>C</u>	C	A	T		4.3 (3)
38	T	G	G	A	T	A	G	<u>A</u>	A	T		8.2 (5)
99	T	G	G	A	T	A	G	C	<u>G</u>	T		5.7 (3)
100	T	G	G	A	T	A	G	C	<u>T</u>	T		3.3 (3)
101	T	G	G	A	T	A	G	C	A	<u>A</u>		<2.0 (3)
Multiple point												
86	<u>A</u>	<u>C</u>	G	A	T	A	G	C	A	T		98 (3)
103	T	G	G	A	T	A	G	C	<u>T</u>	<u>A</u>		70 (3)
104	T	G	G	A	T	A	G	C	<u>G</u>	<u>C</u>		21 (4)
67	T	G	G	A	T	A	G	C	<u>C</u>	<u>G</u>		12 (6)
66	<u>A</u>	<u>C</u>	<u>C</u>	A	T	A	G	C	A	T		6.3 (3)
68	<u>A</u>	<u>C</u>	<u>C</u>	A	T	A	G	C	<u>C</u>	<u>G</u>		<2.0 (2)
21	<u>A</u>	<u>C</u>	<u>C</u>	T	T	A	G	C	A	T		<2.0 (3)
22	T	G	G	A	<u>G</u>	<u>C</u>	<u>T</u>	C	A	T		<2.0 (3)
23	T	G	G	A	T	A	G	<u>A</u>	<u>C</u>	<u>G</u>		<2.0 (3)
249	T	G	G	A	<u>C</u>	A	<u>A</u>	C	A	<u>G</u>		3.5 (2)
250	T	G	G	A	<u>C</u>	A	<u>A</u>	T	A	T		4.5 (2)
248	T	G	G	A	<u>C</u>	A	<u>A</u>	<u>T</u>	<u>C</u>	T		<2.0 (2)
Spacing												
69	T	G	G	A	T	A	G	C	A	— ^c		<2.0 (2)
30	T	G	G	A	T	A	G	C	A	T	A	<2.0 (4)

^a ± indicates a change in both positions. The mutated bases in each oligonucleotide are underlined and in bold type.^b The original equivalent 19-mer oligonucleotides with A at position 7 formed aberrant self-annealed structures and could not be used.^c —, Base is missing at this position.

4% activity each), the combination of TA for AT at positions −2 and −1 (no. 103) restored binding to 70% of wild-type levels. Obviously, the interactions here are both very subtle and flexible and are not fully predictable from the single point mutations. Additional evidence that substitutions which individually did not impair binding could collectively reduce binding to minimal levels was provided by oligonucleotides 248, 249, and 250 (Table 1). In these examples, changes to C at position −6 and A at −4 plus G at −1 (no. 249), T at −3 (no. 250), or T at −3 and C at −2 (no. 248) collectively reduced binding to less than 5% of wild-type,

despite the fact that none of these changes were harmful as individual substitutions.

Methylation interference analysis of the dyad symmetry region. The oligonucleotide binding studies indicated that positions +3 to +8 and −3 to −8 of the consensus binding site were particularly sensitive to transversion mutations. To substantiate the importance of this region of the binding site, EBNA-1 binding to the dyad symmetry region was analyzed after partial methylation of the DNA. In this methylation interference assay, an end-labeled *EcoRI-EcoRV* fragment of pDH144 containing the dyad symmetry region (nucleo-

FIG. 4. Effects of single and multiple alterations within the consensus binding site. (a and b) Autoradiographs of a gel retardation assay performed with 28K EBNA-1 and Raji EBNA-1, respectively. Symmetric 30-mer oligonucleotides were incubated with EBNA-1, and the binding reaction mixture was electrophoresed through a 10% polyacrylamide gel. WT, Wild-type binding site; 7-10, 4-6, and 1-3 contain multiple alterations at the indicated positions; AT, a 2-bp AT insertion between positions +1 and -1; B, bound probe; F, free probe. Probes -1 through -10 contain single transversions at the indicated positions. Note that the 80K Raji EBNA-1 bound to the 30-mer oligonucleotide probe did not enter the gel. (c) Quantitative comparison of the 28K EBNA-1 (■) and Raji EBNA-1 (□) binding data.

TABLE 2. Summary of occurrence and binding data for all naturally occurring EBV half-sites

Location	Position no. ^a											Observed binding affinity	Oligonucleotide no.
	±10	±9	±8	±7	±6	±5	±4	±3	±2	±1			
Family of repeats													
15 times	N	G	G	A	T	A	G	C	A	T	Strong	20	
14 times	N	G	G	G	T	A	G	C	A	T	Strong	70	
7 times	N	G	G	G	T	A	G	<u>T</u>	A	T	Strong	74	
4 times	N	G	G	A	T	A	G	C	<u>C</u>	T	Strong	39	
Dyad symmetry site													
Outside (site 4)	T	<u>C</u>	G	A	T	A	G	C	A	T	Intermediate	86	
	<u>C</u>	G	G	<u>A</u>	A	G	C	A	T			71,137	
Inside (site 3)	<u>G</u>	<u>T</u>	<u>A</u>	A	T	A	<u>A</u>	C	A	T	Weak	32,135,73	
	T	<u>C</u>	<u>A</u>	A	T	A	G	C	A	T		86,135	
Inside (site 2)	T	G	G	A	T	A	G	<u>T</u>	A	T	Weak	74	
	<u>G</u>	<u>T</u>	<u>A</u>	G	T	A	G	<u>T</u>	A	T		32,135,74	
Outside (site 1)	<u>C</u>	G	G	G	<u>A</u>	A	G	C	A	T	Intermediate	71,137	
	<u>C</u>	G	G	G	T	A	G	C	A	T		70,137	
Q locus													
Site 2	<u>G</u>	G	G	A	T	A	G	C	<u>G</u>	T	Very weak	99	
	<u>C</u>	<u>C</u>	G	G	T	A	G	C	<u>G</u>	<u>C</u>		86,99,102	
Site 1	<u>C</u>	G	G	G	T	A	<u>A</u>	<u>T</u>	A	<u>C</u>	Very weak	73,74,102	
	A	G	G	A	<u>G</u>	A	G	C	A	T		35	
Family of repeats site 21	T	G	G	G	T	A	<u>T</u>	C	A	T	Nil	37	
	<u>C</u>	<u>A</u>	G	A	<u>C</u>	A	G	C	A	T		136,75,137	

^a ± indicates either position. Bases that differ from the predominant family of consensus repeats are underlined and in bold type.

FIG. 5. Summary of the results of the binding site analyses with mutated oligonucleotides and correlation with the naturally occurring EBNA-1-binding sites. (Top) Variations in composition of half-palindromic EBNA-1-binding sites in the EBV genome. Symbols and abbreviations: ■, a particular nucleotide occurs frequently at either the plus or minus position or both within the family of repeats; ■, occurs infrequently in the family of repeats; Q, occurs only in the Q locus; DS, occurs only in the dyad symmetry; DS/Q, occurs in both the Q locus and the dyad symmetry but not in the family of repeats; "21," occurs only in site 21. (Bottom) Affinity of synthetic symmetric mutant oligonucleotides for EBNA-1 in gel retardation assays. Symbols: ■, a particular nucleotide at this position within the standard consensus half-palindromic sequence gives high-affinity binding, i.e., 70 to 150% of wild type; ■, weak affinity, 20 to 40% of wild type; —, minimal affinity, 2 to 8% of wild type.

tides 8993 to 9133 of the B95-8 genome) was partially methylated by using dimethyl sulfate and incubated with 28K EBNA-1. A mobility retardation assay was performed, and the fully shifted and unshifted DNA was isolated from the gel and sequenced chemically (Fig. 6). The underrepresentation of particular guanine nucleotides in the shifted species indicates that methylation of these bases interferes with binding. Strong interference with EBNA-1 binding occurred with methylation of the guanines at positions -7 and -8 in all four sites, whereas there was minimal interference at -9 and no observed interference at the -4 guanine. Therefore, contacts on this half of the palindrome occur at positions -8 and -7 and do not extend beyond position -5. On the other half of the palindrome methylation resulted in weak interference at the +3 guanine and no interference at the guanines at +8 (binding site 3) or +10 (binding site 4). When the positions of these contacted guanine residues are displayed in the context of the double-stranded consensus binding site (Fig. 6), it can be seen that the predicted contact points on each strand lie exactly one turn of the helix (10 bp) apart. The contact regions predicted by the methylation interference data, positions -8 to -3 and +3 to +8, are completely consistent with the binding data from the transversion mutation experiments with the synthetic oligonucleotides.

EBNA-1 binding affinity. A Scatchard analysis of binding data was used to determine the equilibrium dissociation constant (K_d) of the EBNA-1 fusion protein for the consensus synthetic oligonucleotide binding site. Binding reactions were performed with a constant amount of 28K EBNA-1, a constant amount of 32 P-labeled oligonucleotide (1 fmol), and increasing concentrations of unlabeled oligonucleotide. The reaction mixtures were subjected to gel electrophoresis, and the shifted and unshifted bands were excised for radioactivity measurements. The results of this assay were plotted as bound DNA versus bound DNA/free DNA (Fig. 7). The inverse of the slope of the line gives the K_d , the equilibrium dissociation constant for the protein-DNA interaction. From

Scatchard analysis the apparent dissociation constant of 28K EBNA-1 for the consensus oligonucleotide binding site was estimated to be 1.5×10^{-11} M. A similar analysis using human placental DNA as a competitor (results not shown) indicated that the affinity of EBNA-1 for nonspecific DNA was 7 orders of magnitude lower than its affinity for specific binding sites.

DISCUSSION

Maintenance of EBV latent infection is dependent on EBNA-1 for replication of the latent episomal form of EBV DNA and for transactivation of expression from the *Bam*HI-C latency promoter. Both of these functions are mediated through binding of EBNA-1 to its cognate recognition sequences. Given the central role of DNA binding for the biological activity of EBNA-1, a better understanding of the factors that determine binding specificity is of some interest. The three EBNA-1-binding loci within the EBV genome contain a total of 26 individual binding sites, and the family of repeats, the dyad symmetry, and the Q locus each show differing affinities for the EBNA-1 protein (7). The major goal of this study was to determine the basis of these affinity differences and to define the exact sequence requirements for EBNA-1 binding by mutational analysis.

All of the sequence variations that occur naturally within the family of repeats showed high-affinity binding in the oligonucleotide assays, indicating that all of the binding sites within the family are of essentially equal affinity. This observation is compatible with earlier DNase I footprinting studies (13) which did not show any differences in the pattern of protection between individual binding sites within the family of repeats. However, an adjacent repeat unit (site 21) did not bind EBNA-1 in the footprinting study. Site 21 has a G to T substitution at position -4 and a T to C substitution at +6 (Table 2). In the oligonucleotide experiments the G to T substitution at position 4 eliminated binding, and therefore this variation presumably explains the lack of binding to site

FIG. 6. Methylation interference analysis of EBNA-1 binding to the dyad symmetry region. An *EcoRI-EcoRV* fragment containing the dyad symmetry locus was end labeled at the *EcoRI* site and treated with dimethyl sulfate prior to incubation with 28K EBNA-1. Bound and unbound DNA was isolated from a 5% polyacrylamide gel, cleaved with piperidine, and electrophoresed on a 6% sequencing gel. Symbols: ◐ and ●, guanine residues that interfere with binding; ◑ and ◒, partial interference; ◓ and ◔, no interference. The locations and sequence of the four EBNA-1-binding sites within the dyad symmetry are indicated. Note that the contact guanines on each DNA strand (*) lie 10 bp apart.

21. The other changes in site 21 at positions +6, +9, and +10 would not be expected to be contributing factors individually but might be so in combination with the change at position -4 in the other half-site.

In filter-binding studies, the intact dyad symmetry locus has lower overall affinity for 80K EBNA-1 than either the family of repeats or DNA fragments containing monomer or dimer consensus sites (7). Within the dyad symmetry region itself, the outside pair of sites (sites 1 and 4) showed equal affinity to each other in the DNase footprinting studies of Rawlins et al. (13). Each of these sites has a T to A substitution in one half-site at position 6, which in the oligonucleotides gave an intermediate affinity (30 to 50% of wild type). Again, changes at the 9 and 10 positions are assumed not to be contributory. The situation with the two inner sites of the dyad symmetry is interesting. On the basis of DNase I footprints, sites 2 and 3 are equal to each other in affinity but have a lower affinity than sites 1 and 4. The substitutions in these inner sites (G to A at positions -4, -8,

and +8 of site 3, C to T at positions -3 and +3 and G to A at position +8 of site 2) each gave high-affinity binding when introduced individually into the oligonucleotides. However, we also demonstrated in the oligonucleotide studies that changes which were individually acceptable could collectively reduce binding to minimal levels. Oligonucleotides 249 and 250, although not identical to the dyad symmetry sites, contain similar types of substitutions. Oligonucleotide 249 contains the G to A change at position 4 along with individually acceptable changes at positions 1 and 6, while oligonucleotide 250 carries the C to T substitution at position 3 along with individually acceptable changes at positions 4 and 6. Nevertheless, binding to these substrates was only 3.5 and 4.5% of wild-type levels. Thus, we propose that it is the combination of substitutions that results in the reduced affinity of the inner sites of the dyad symmetry. As a corollary, we suggest that a combination of tolerated changes within a half-site can be more detrimental to binding than an individual less tolerable substitution. The overall

FIG. 7. Scatchard analysis of 28K EBNA-1 binding. Binding reactions were performed with constant amounts of 28K EBNA-1 extract and labeled oligonucleotide and an increasing concentration of unlabeled oligonucleotide binding site. Binding reaction mixtures were subjected to electrophoresis on a 10% polyacrylamide gel and, after autoradiography, the shifted and unshifted bands were excised and counted. The data were plotted according to equations 1 (A) and 2 (B) in Materials and Methods. The calculated dissociation constant for 28K EBNA-1 is 1.5×10^{-11} M.

reduced binding affinity of the dyad symmetry locus can therefore be explained by base substitutions within this locus. Secondary structure formation may be a feature of this locus, and conformation could also affect the binding of EBNA-1, although to what extent is presently unclear.

The Q locus displayed the lowest affinity for 80K EBNA-1 in our previous filter-binding studies (7). The gel retardation experiment showed that both potential sites in Q do in fact bind EBNA-1. Site 2 of the Q locus has a symmetric A to G substitution at position 2 which results in minimal binding (5% of wild type) in the oligonucleotides. Possibly the introduction of the paired substitution, A to G at position 2 together with T to C at position 1 in the other half of this binding site, ameliorates the negative effect somewhat, since oligonucleotide 104 carrying this double substitution had a higher affinity (21% of wild type). Site 1 of the Q locus would also be expected to be seriously impaired in its binding to EBNA-1 on the basis of the oligonucleotide data. One half of this site has a T to G substitution at position 6 which results in minimal binding in the oligonucleotides. The other half of this site carries a triple substitution of individually acceptable bases, G to A at position 4, C to T at position 3, and T to C at position 1. Oligonucleotide 250, which has the adenine and thymidine substitutions at positions 4 and 3 and an individually acceptable T to C change at position 6, has minimal binding (4.5% of wild type). Overall, the oligonucleotide mutational studies provided experimental evidence that the major differences in the relative affinities of the family of repeats, the dyad symmetry, and the Q locus arise primarily from the variations in base sequence that occur within the individual binding sites in these loci. Our suggested consensus high-affinity recognition site for EBNA-1 is the sequence 5'-GRTAGCNNNGCTAYC-3', although it is clear that certain combinations of nucleotides at the central four positions are preferred.

We also obtained some information on the interaction between EBNA-1 and its binding site. Since the binding site is palindromic it seemed likely that EBNA-1 would bind as a dimer, and biochemical studies indicate that EBNA-1 exists

as a dimer in solution (R. F. Ambinder, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1989). The fact that the spacing of the halves of the palindrome was critical for binding, taken together with the methylation interference data showing contact points on each strand 10 bp apart, is consistent with a symmetric dimer complex encompassing two adjacent turns of the helix, with each subunit making contacts with parts of both strands across the major groove. A similar conclusion was recently drawn by Kimball et al. (8) from hydroxyl-radical footprinting and methylation protection studies, although in contrast to our data the latter studies were interpreted to indicate contacts at position -3 also. All of these observations are consistent with the predicted presence of a helix-turn-helix DNA-binding moiety within the EBNA-1 protein, although there is no experimental or genetic evidence for such a feature available at present.

Sixteen of the twenty binding sites within the family of repeats and all four of the dyad symmetry sites in the EBV genome have the sequence ATAT within the central axis of the palindrome (positions -2, -1, +1, and +2). This conservation initially seemed surprising, given the facts that no protein contacts occur at these positions according to hydroxyl-radical footprinting (8) and that many point mutations were tolerated in our oligonucleotides. The approximately fivefold preference for alternating adenine and thymidine (ATAT or TATA) over GCGC and CGCG in the oligonucleotides may reflect conformational requirements of the binding site related to A · T pairs having greater flexibility than G · C pairs. On the other hand, the combination AATT or TTAA at these positions almost totally disrupted binding. Conceivably, the absolute preference for ATAT over TATA in the naturally occurring sites may reflect conservation of the recognition site for some unknown cellular factor rather than being relevant for EBNA-1 binding.

The adjacent binding sites in the family of repeats are predominantly 30 bp apart, and in the dyad symmetry region they are 21, 32, and 21 bp apart, respectively. This would result in both cases in binding of successive protein complexes to adjacent sites along the same face of the DNA helix. In contrast, the previously predicted sites of the Q locus, which we now know both represent valid binding sites, are 24 bp apart and therefore in this locus the two adjacent EBNA-1 complexes would be expected to be located on opposite sides of the helix. The functional consequences, if any, of these juxtapositions are unknown. Something about the structure of the region I tandem repeats appears to result in termination of replication forks (6), whereas there is evidence that the dyad symmetry region acts as an initiation site for plasmid replication (5, 6, 23). The Q locus is apparently not required for either lytic or plasmid DNA replication. Furthermore, its location in the EBV genome does not suggest any obvious function for the Q binding sites other than as a potential negative autoregulatory mechanism involving blocking the elongation of latency transcripts from proceeding beyond EBNA-2 and into the EBNA-1-coding region.

The dissociation constant for 28K EBNA-1 was calculated from Scatchard analysis to be 1.5×10^{-11} M, and the K_d for the complete EBNA-1 protein is of the same order of magnitude (D. Rawlins, unpublished results). This value is very similar to that for other replication-associated proteins. Simian virus 40 T antigen (18) has a dissociation constant of between 1×10^{-11} and 5×10^{-11} M, and the cellular DNA-binding protein NF1, which is involved in adenovirus

DNA replication, also has a dissociation constant of 2.0×10^{-11} M (16).

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