JOURNAL OF VIROLOGY, Aug. 1993, p. 4875–4885
0022-538X/93/084875-11$02.00/0

Vol. 67, No. 8

Separation of the Complex DNA Binding Domain of EBNA-1 into DNA Recognition and Dimerization Subdomains of Novel Structure

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Received 28 January 1993/Accepted 15 May 1993

EBNA-1 is essential for replication of the latent episomal form of the Epstein-Barr virus genome and is involved in regulation of viral latency promoters. EBNA-1 activity is mediated through direct DNA binding. The DNA binding and dimerization functions of EBNA-1 have previously been located to a carboxy-terminal domain, amino acids (aa) 459 to 607. To identify and define the subdomains for these two functions, we created an extensive series of deletions and point mutations in an EBNA-1 (aa 408 to 641) background. The ability of the EBNA-1 mutants to heterodimerize with a wild-type EBNA-1 (aa 459 to 641) polypeptide was tested in immunoprecipitation assays with a monoclonal antibody, EBNA.OT1x, that recognizes EBNA-1 (aa 408 to 641) but not EBNA-1 (aa 459 to 641). These experiments revealed that mutations affecting dimerization occurred over two separate regions, aa 501 to 532 and aa 554 to 598. DNA binding was tested in mobility shift assays against a panel of oligonucleotide-binding sites. Dimerization was a prerequisite for DNA binding. The DNA recognition domain was localized to a separate region, aa 459 to 487, upstream of the dimerization domain. EBNA-1 variants carrying substitutions at aa 467 and 468 and at aa 477 gave a pattern of binding to mutant oligonucleotide probes that implicates these particular amino acids in DNA recognition. EBNA-1 appears to utilize novel mechanisms for both DNA recognition and dimerization since neither domain conforms to previously described structural motifs.

The EBNA-1 protein has a central role in the maintenance of a latent Epstein-Barr virus infection. EBNA-1 is the only virus-encoded protein required for replication of the episomal form of the Epstein-Barr virus genome (39, 41). The latency origin of replication, *oriP*, contains two EBNA-1-binding loci: region I, the family of repeats, consists of 20 tandem copies of the EBNA-1-binding site, and region II, the dyad symmetry region, contains two pairs of overlapping, lower-affinity, binding sites (15, 24, 26). The dyad symmetry region is the site of initiation of DNA replication (9) while the family of repeats provides an enhancer function (25, 38). Electron microscopic studies have indicated that EBNA-1 molecules bound to these two elements of *oriP* can mediate looping out of the intervening DNA sequences, a process that may facilitate formation of the replication initiation complex (8, 35). An additional role for EBNA-1 in nuclear matrix attachment during replication has been postulated (14). EBNA-1 also participates in regulation of latency gene transcription. EBNA-1-mediated transactivation of the latency C promoter has been described previously (36), and immediately downstream of the latency F promoter are two EBNA-1-binding sites which may negatively modulate transcription from this promoter (2, 28, 29, 31, 33).

The replication and gene regulation functions of EBNA-1 are mediated through DNA binding (15, 24). EBNA-1 binds DNA as a dimer (1, 7), and both the DNA binding and dimerization functions are located in the carboxy-terminal third of the 641-amino-acid (aa) protein (1, 13, 24). Mapping studies using amino- and carboxy-terminal deletions of this segment of the EBNA-1 open reading frame placed the DNA binding and dimerization domains between aa 459 and 607 (1). The relative positioning of these two functional domains and their nature have been a matter of conjecture. An amino acid sequence comparison led Inoue et al. (13) to suggest that EBNA-1 contained a basic helix-loop-helix (bHLH) domain. The nature of the dimerization domain is of some relevance since certain motifs, such as the helix-loop-helix motif, allow heterodimerization with other proteins of the same class. Such interactions can significantly modify the function of the individual proteins involved.

In a previous attempt to separate the DNA-binding and dimerization domains, we created a set of relatively large internal deletions across the aa 459 to 607 region (32). However, these deletions all abolished DNA binding and greatly reduced dimerization, indicating that the entire region spanning aa 459 to 607 was very sensitive to structural perturbation. In the present study, we sought to minimize nonspecific effects on EBNA-1 structure by introducing a series of amino acid substitutions and small five- to seven-aa deletions into the segment of EBNA-1 from aa 459 to 607. These mutants plus the availability of a new monoclonal antibody against EBNA-1, EBNA.OT1x, allowed us to separate the individual DNA binding and dimerization domains and to gain some insight into amino acids that may be involved in DNA recognition. Interestingly, EBNA-1 does not appear to belong to any previously recognized class of DNA-binding protein.

MATERIALS AND METHODS

Plasmid constructions. Wild-type EBNA-1 (aa 408 to 641) [referred to hereafter as EBNA-1(408-641)] was expressed by in vitro transcription and translation from pRA362 (1) and

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EBNA-1 (aa 459 to 641) [referred to as EBNA-1(459-641)] was generated similarly from pWS61 (32). The deletion series pRA374(479SRΔ481-520), pRA376(499SRΔ501-20), pRA378(Δ525-32), pWS52A(Δ537-53), pWS43(555SRΔ557-64), pWS46(576SRΔ578-616), and pWS47(Δ599-614,615SR) has been described previously (32), as have EBNA-1 constructions containing unique *Xba*I sites at codons 479 and 480, 499 and 500, 521 and 522, 555 and 556, and 615 and 616. Three of the *Xba*I mutations were transferred into the in vitro transcription vector pGH253 to create pMRC32 (479SR), pMRC30 (521S), and pMRC31 (576SR).

The oligonucleotides LRA27 (5′-CCGGATCCTGTAGGG GAAGCCGAT), which anneals to the EBNA-1 template at codon 408, and LGH312 (5′-GGCCCTGCAGTCACTCCTG CCCTTCCTC), which anneals to the EBNA-1 template at codon 641, served as the outside primers for polymerase chain reaction (PCR) mutagenesis. Recombinant PCR with these outside primers and a pair of overlapping inside primers was used to generate pMRC35 (467VD) containing a *Sal*I site at codons 467 and 468 and pMRC36 (511VY) containing an *Acc*I site at codons 511 and 512. Briefly, the oligonucleotides LRA27 and 5′-CACGGTCGACTCCAAAC CACCCTCC were used to amplify codons 408 to 473 and introduce mutated sequences at codons 467 and 468, and oligonucleotides 5′-TGGAGTCGACCGTGGTCAAGGAGGT and LGH312 were used to amplify codons 462 to 641 with mutations at 467 and 468. The two PCR products were then purified, denatured, annealed, and again amplified with the outside primers LRA27 and LGH312 and cloned into pGH253 to create pMRC35. Similarly, plasmid pMRC36 was generated with the primer pairs LRA27 and 5′-TACTGTA TACATATACGAACACACC to amplify codons 408 to 517 with mutations at codons 467 and 468, and oligonucleotides 5′-ATATGTATACAGTAAGACCTCCCTT and LGH312 were used to amplify codons 506 to 641 with mutations at 511 and 512. A similar strategy was also used to generate pMRC42(513A). The outside primers for pMRC42 were LRA27 and LRA28, 5′-CGGATCCTGCAGGAATTCCAAT TGCCCCATGTTGGTA (which anneals outside the EBNA-1 open reading frame), and the internal primer pair was 5′-GGAGGTCTTAGCACCTCCATA and 5′-TATGGAGGT GCTAAGACCTCC.

The second set of small deletions were created by using forward PCR primers containing *Xba*I, *Sac*I, or *Sal*I restriction enzyme sites to loop out the desired codons. The PCR products obtained with these primers and LGH312 were then used to replace the equivalent wild-type DNA fragment. The PCR primers, with restriction sites underlined, were 5′-GCTATCTAGATTTTTACAAACTCATATA, pMRC 45 (555SRΔ557-64); 5′-ATTTTCTAGAAGAGCTCTCCTG-GCTAGG, pMRC46 (479SRΔ481-5); 5′-TACCTCTAGATT CGTATATGGAGGTAGT, pMRC47 (499SRΔ501-7); 5′-TT AAGAGCTCTCGTAGAAAGGACTACC, pMRC49 (Δ489-93); and 5′-TGGAGTCGACTCCAACCCGAAATTT, pMRC 57A1 (467VDΔ469-73). Plasmid pMRC57A2 (467VDΔ469-73,478V) contains the same deletion as pMRC57A1 plus a spontaneous mutation (aa 478, F to V). EBNA-1(468-641) (pMRC53) was made by moving the *Sal*I-*Pst*I DNA fragment from pMRC35 into an in vitro transcription vector.

Utilizing the introduced *Xba*I sites or the native *Sac*I site in the EBNA-1 gene and mismatched PCR primers, the following additional point mutations were created: pMRC24 (485D; reverse primer: 5′-CAGGAGAGCTCTATCACCTT CTGCAATG; *Sac*I); pMRC26 (477A-SR; reverse primer 5′-CAATTCTAGAAAATGCCGGGTTGGAACC; *Xba*I at 479 and 480); pMRC28 (574DD; reverse primer 5′-AAT CGCTCTAGAATCATCCTCAGCAAATAT; *Xba*I at 576 and 577); pMRC29 (517AAASS; reverse primer 5′-AGTTCC TCTAGATGAGGGGGCAGCGGAGGTCTTACT; *Xba*I at 521 and 522); pMRC33 (488R; 5′-TTAAGAGCTCGCCTG GCTAGGAGT; *Sac*I site); and pMRC56 (490P; 5′-TTAA GAGCTCTCCTGCCCAGGAGTCACGTA, *Sac*I site). A pair of oligonucleotides, 5′-CTAGAGGAACTTGGGTCC ACT and 5′-CTAGAGTGGACCCAAGTTCCT, were annealed and cloned into the *Xba*I site of pMRC47 to create pMRC50 (499SR505HSR). Cloning in the reverse orientation created pMRC51 (499SRSVAPSSS). All the mutations were confirmed by double-stranded dideoxy sequencing. With the exception of the one case noted, no spontaneous PCR-generated alterations were observed.

In vitro transcription-translation. Plasmid DNA was linearized downstream of the coding sequence of EBNA-1 and incubated with T7 RNA polymerase to prepare capped mRNA by using an in vitro transcription kit (Stratagene, La Jolla, Calif.). In vitro translation was carried out by using rabbit reticulocyte lysates (Promega, Madison, Wis.). A standard in vitro translation reaction contained 1 to 2 μg of mRNA in a 50-μl reaction mixture and 50 μCi of [^{35}S]methionine (800 Ci/mmol) purchased from New England Nuclear (Wilmington, Del.). Cotranslations contained equal amounts of the two different RNAs. Labeled proteins were stored at −70°C.

Immunoprecipitation assays using EBNA.OT1x. The monoclonal antibody EBNA.OT1x specifically recognizes an epitope between aa 408 and 459 of EBNA-1 (19a). Two microliters of each in vitro-translated protein was diluted to 100 μl with TSET buffer (150 mM NaCl, 50 mM Tris-HCl, 0.1 mM EDTA, 2% Triton X-100 [pH 8.0]), and 2 μl of EBNA.OT1x hybridoma culture supernatant was added. The mixture was incubated at 4°C for 1 h. Five milligrams of protein A-Sepharose (in TSET) was added, and after 30 min of incubation at 4°C, the precipitate was pelleted and washed once in TSET and three times in NET (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.1 mM EDTA). The sample was resuspended in buffer (62.5 mM Tris [pH 6.8], 10% glycerol, 4% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 0.01% bromophenol blue) and boiled for 3 min before electrophoresis on an SDS–13% polyacrylamide gel.

Preparation of oligonucleotide-binding substrates. Symmetric consensus and mutant binding site probes were prepared from 19-mer synthetic single-stranded oligonucleotides which could self anneal and thus serve as primers for a Klenow fill-in reaction, generating double-stranded 30-mers (2). The oligonucleotide-binding sites used were 20, 33, 36, 71, 73, 74, 96, 97, 100, 102, 104, 196, and 197, as described in Ambinder et al. (2). Oligonucleotides (10 pmol) were heated to 75°C and slowly cooled to room temperature for self annealing. [^{32}P]dATP (10 μCi), and 10 nmol each of dCTP, dGTP, and dTTP was added to a 20-μl reaction, along with 5 U of Klenow polymerase. After 1 h of incubation, the reaction was stopped by adding 45 μl of STE (150 mM NaCl, 10 mM Tris-HCl, 0.1 mM disodium EDTA [pH 7.4]), and the DNA was purified in a G-25 spin column (5 Prime → 3 Prime, Inc., Boulder, Colo.).

Electrophoretic mobility shift assays. In vitro-translated EBNA-1 was incubated with probe DNA (20 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 $MgCl_2$–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.). For supershift assays, 2 μl of 1:10 diluted monoclonal

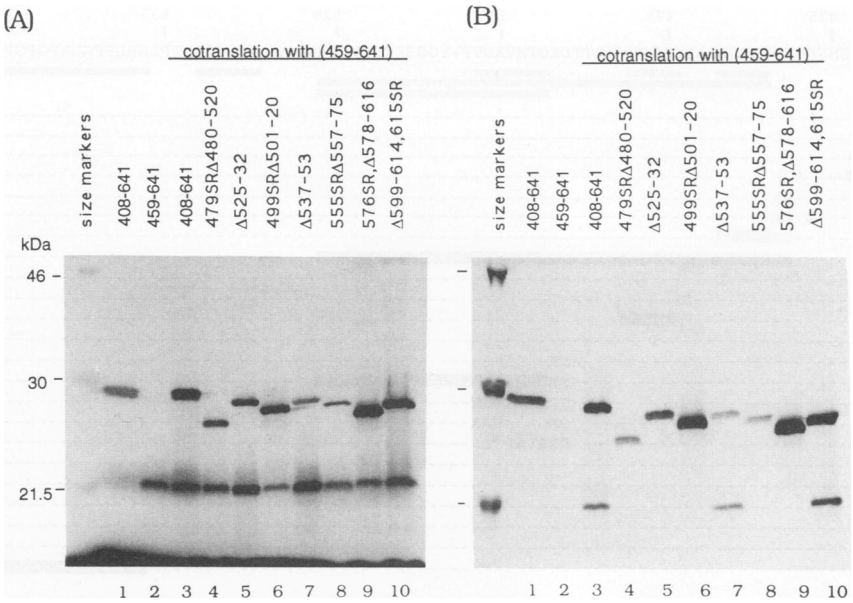

FIG. 1. Use of the monoclonal antibody EBNA.OT1x to assess EBNA-1 dimerization. (A) In vitro-translated EBNA-1 polypeptides were analyzed by SDS-PAGE on a 13% gel. Each lane contains 3 μl of the in vitro translation mixture. Lanes 1, wild-type EBNA-1(408-641); 2, wild-type EBNA-1(459-641); 3, cotranslation of EBNA-1(408-641) and EBNA-1(459-641); 4 to 10, cotranslation of EBNA-1(459-641) with a previously described set of EBNA-1 deletions (31). (B) Immunoprecipitation of the in vitro-translated polypeptides shown in panel A with monoclonal antibody EBNA.OT1x. EBNA-1(408-641) was immunoprecipitated by the antibody (lane 1) but EBNA-1(459-641) was not (lane 2). Cotranslation of EBNA-1(459-641) with EBNA(1408-641) resulted in the formation of a heterodimer, and EBNA-1(459-641) was coimmunoprecipitated with EBNA.OT1x (lane 3). Lanes 4 to 10, immunoprecipitation after cotranslation of the indicated EBNA-1 deletions with EBNA-1(459-641).

antibody (EBNA.OT1x) culture supernatant was added to the mixture and incubated for 15 min at room temperature. The reaction mixtures were loaded onto a 6% polyacrylamide gel in HEE buffer (10 mM HEPES [pH 7.5], 1 mM EDTA, 5 mM EGTA) and electrophoresed at 10 V/cm at room temperature for 1.5 h. The gels were dried and autoradiographed.

RESULTS

Use of the monoclonal antibody EBNA.OT1x to assay for EBNA-1 dimerization. Both the DNA binding and dimerization domains of EBNA-1 map to a region, aa 459 to 607, at the carboxyl end of the protein (1). In a previous attempt to separate the domains for these two functions, a set of large internal deletions across this region was tested for dimerization ability by using glutaraldehyde cross-linking (32). However, each of the deletions had severely compromised dimerization ability in this assay. Thus, a different approach was required to identify sequences involved in EBNA-1 dimerization. Here we report the use of an EBNA-1-specific monoclonal antibody and a new series of mutations that made it possible to dissect regions of the protein required for dimerization.

EBNA.OT1x is a monoclonal antibody raised against an EBNA-1 protein that lacked the Gly-Gly-Ala repeats. The antibody recognizes an epitope located between aa 408 and aa 459 of EBNA-1 (19a). When this antibody was used to immunoprecipitate different in vitro-translated EBNA-1 polypeptides, we found that EBNA.OT1x immunoprecipitated in vitro-translated EBNA-1(408-641) (Fig. 1B, lane 1) but not EBNA-1(459-641) (Fig. 1B, lane 2). When cotranslated, EBNA-1(408-641) formed a heterodimer with the polypeptide, spanning aa 459 to 641, which could now be coimmunoprecipitated with EBNA.OT1x (Fig. 1B, lane 3). Thus, coimmunoprecipitation of EBNA-1(459-641) could be used to assay for the dimerization ability of deleted or mutated aa 408 to 641 polypeptides. The original set of large internal deletions was cotranslated with EBNA-1(459-641) (Fig. 1A, lanes 4 to 10). The cotranslated polypeptides were then immunoprecipitated with EBNA.OT1x (Fig. 1B, lanes 4 to 10). In this more sensitive assay, two of the deletions (Fig. 1B, lanes 7 and 10) were able to heterodimerize with EBNA-1(459-641), indicating that aa 537 to 553 and 599 to 614 are dispensable for dimerization.

Identification of domains essential for EBNA-1 dimerization. To further localize the EBNA-1 dimerization domain, we constructed a series of small deletions and point mutations in an EBNA-1(408-641) background (Fig. 2). Deletions are named with a Δ symbol followed by the number of the first and last amino acid deleted. For point mutations, the number of the first amino acid altered is given followed by the single letter symbol for the introduced amino acid. Many of the mutations were created within EBNA-1-coding sequences carrying a unique *Xba*I restriction site, which itself introduces a serine and arginine (SR) mutation. (Note that the *Xba*I site at codons 521 and 522 introduces only a serine because the wild-type aa 522 is arginine). None of the SR mutations affected dimerization ability (Fig. 3B and C and data not shown). The new series of EBNA-1 mutants were cotranslated in vitro with EBNA-1(459-641) and immunoprecipitated with EBNA.OT1x. The single exception to this protocol was EBNA-1(468-641), which lacks the epitope for the EBNA.OT1x antibody and was cotranslated with EBNA-1(408-641). The results obtained with deletions and grouped mutations are presented in Fig. 3A and B and with point mutations predominantly in Fig. 3C. Each of the

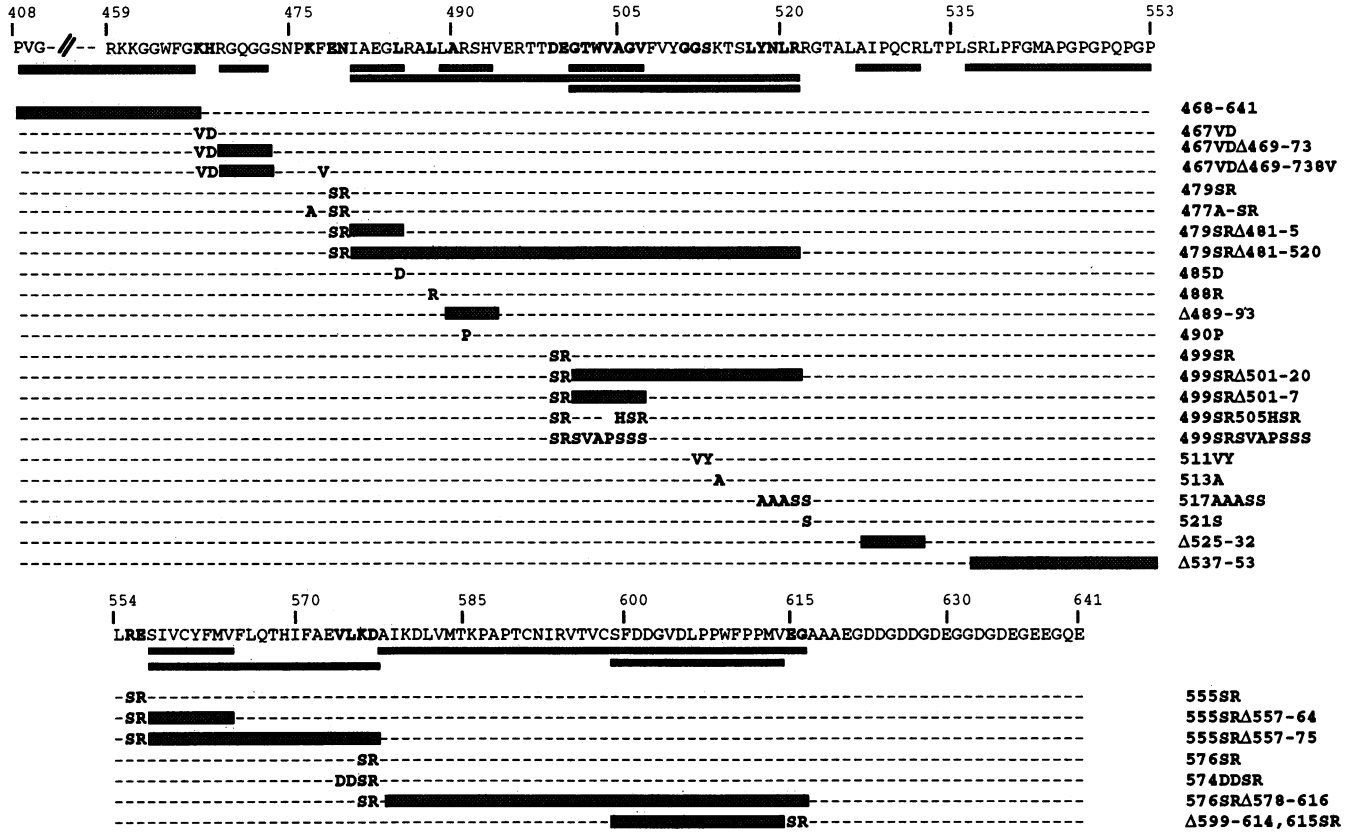

FIG. 2. Structure of the deletions and point mutations used in this study. The amino acid sequence of EBNA-1(408-641) is shown, with the amino acids subjected to mutagenesis indicated in bold type and the positions of deletions indicated by the stippled bars. Each mutant polypeptide is named in the right-hand column, and the position and nature of the mutations carried by each is shown.

constructions carrying deletions located between aa 459 and 493 retained the ability to heterodimerize (Fig. 3A, lanes 1, 3, and 4, and 3B, lanes 3 and 5). Point mutations in this segment likewise did not affect dimerization (Fig. 3A, lane 2, and 3C, lanes 5, 6, and 8). We had already shown that a downstream segment encompassing aa 537 to 553 was also dispensable for dimerization (Fig. 1B, lane 7). However, a deletion located between these two regions, 499SRΔ501-7, abolished dimerization ability (Fig. 3B, lane 4). Further, mutation of these seven amino acids (499SRSVAPSSS) or even of aa 505 to 507 (499SR505HSR) also destroyed the ability to dimerize (Fig. 3B, lanes 7 and 6, respectively). Mutation of nonhydrophobic residues immediately downstream at aa 511 and 512 and aa 513 did not affect dimerization ability (Fig. 3C, lane 7, and 3B, lane 1), but mutation of aa 517 to 520, three of which are hydrophobic residues, did abolish dimerization (Fig. 3C, lane 11). An adjacent deletion, Δ525-32, which was part of the previously described deletion set, also lacked dimerization ability (Fig. 1B, lane 5). Therefore, one of the dimerization domains involves residues located between aa 501 and 532.

Heterodimerization assays with the set of large deletions had implicated a second region located between aa 554 and 598 in dimerization function (Fig. 1, lanes 8 to 10). The requirement for residues in this region was confirmed. A small deletion, 555SRΔ557-64, abolished dimerization function (Fig. 3B, lane 2), as did a point mutation, 574 DDSR, which changed two hydrophobic residues (Fig. 3C, lane 10). The dimerization results are summarized in Table 1. We conclude that EBNA-1 has a bipartite dimerization domain, with residues located between aa 501 and 532 and between aa 554 and 598 being essential.

DNA binding by EBNA-1 heterodimers and specificity of EBNA.OT1x. The major goal of this study was to separate the DNA binding domain of EBNA-1 from its dimerization domains. We first tested DNA binding of the new series of mutants in the context of heterodimers. These experiments not only provided information on DNA-binding capability but also provided confirmatory evidence for heterodimerization. We demonstrated that in vitro-translated EBNA-1(408-641) and EBNA-1(459-641) were capable of binding to a ^{32}P-labeled 30-mer oligonucleotide probe in a mobility shift assay (Fig. 4A, lanes 1 and 3). The shifted band generated by binding of EBNA-1(408-641) was supershifted by the addition of the EBNA.OT1x antibody while, as expected, that generated by binding of EBNA-1(459-641) was not (Fig. 4A, lanes 2 and 4). The cotranslated polypeptides spanning aa 408 to 641 and 459 to 641 produced an additional shifted species (Fig. 4A, lane 9) of intermediate mobility, which resulted from the binding of heterodimers containing one subunit each of EBNA-1(408-641) and EBNA-1(459-641). Interestingly, EBNA.OT1x supershifted the aa-408-to-641 homodimer complex completely but was unable to supershift the heterodimer species (Fig. 4A, lanes 6 and 10). The antibody apparently requires two epitopes to form a stable complex with DNA-bound EBNA-1 even though it can interact with, and immunoprecipitate, free heterodimers carrying only a single copy of the epitope. This requirement

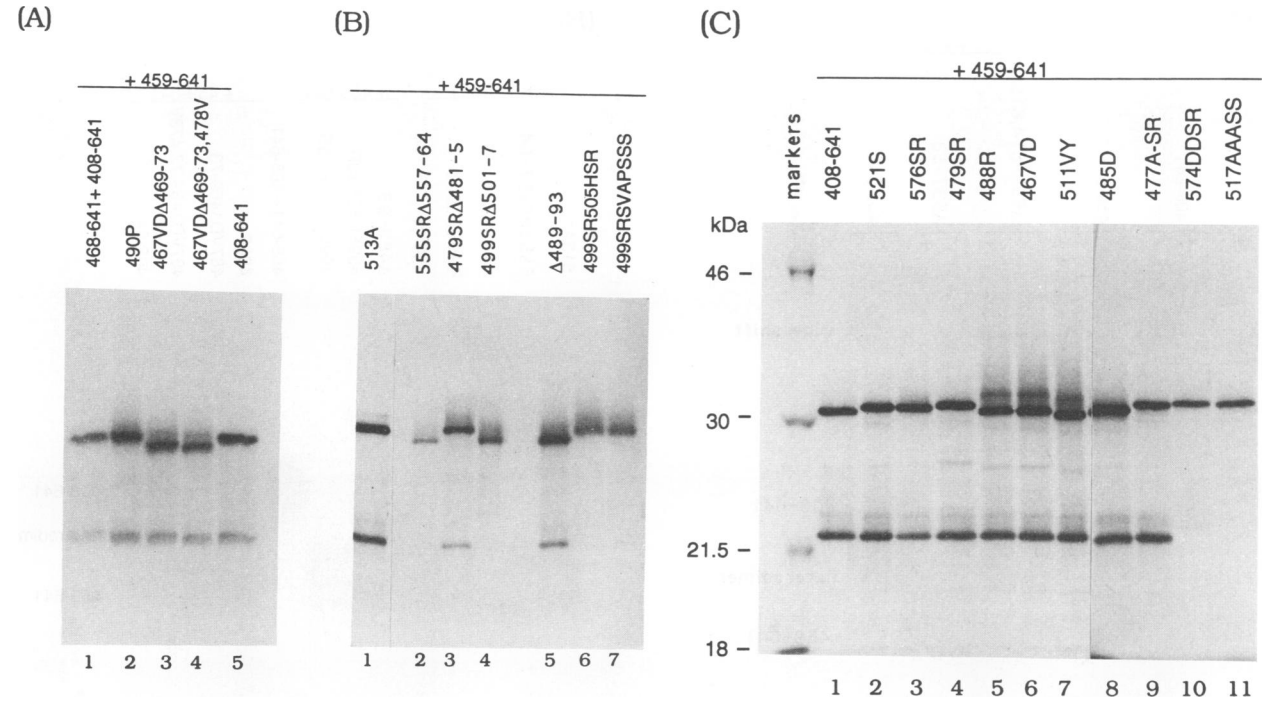

FIG. 3. The effect of small deletions (A and B) and point mutations (C) on EBNA-1 dimerization. Immunoprecipitation by EBNA.OT1x of cotranslated EBNA-1(459-641) and EBNA-1 polypeptides carrying the indicated mutations was performed as described in the legend to Fig. 1.

may be brought about by the change in conformation undergone by EBNA-1 on binding (32). The two original large deletions that could form heterodimers were also cotranslated with EBNA-1(459-641) and tested for DNA binding. Heterodimers between Δ599 to 614 and EBNA-1(459-641) were capable of DNA binding (Fig. 4A, lanes 5 and 6). The Δ537-553 polypeptide remained unable to bind DNA even as a heterodimer (Fig. 4A, lanes 7 and 8). This may reflect a greater perturbation of secondary structure by the aa 537 to 553 deletion.

Separation of the EBNA-1 DNA binding domain from the dimerization domains. We next examined the ability of the series of small deletions and point mutations in EBNA-1(408-641) to bind DNA as heterodimers with EBNA-1(459-641). Dimerization is essential for DNA binding. The mutants that failed to heterodimerize (Fig. 3; Table 1) also failed to bind DNA (Fig. 4B, lanes 2, 4, 7, and 8; Fig. 4C, lanes 13 and 14). The *Xba*I mutations (SR) did not affect DNA binding (Fig. 4C, lanes 2 to 4, and data not shown). Two of the mutations that lay within the boundaries of the first dimerization domain but did not affect dimerization ability, 511VY and 513A, were capable of DNA binding (Fig. 4C, lane 7, and 4B, lane 1). Of greatest interest were the four constructions carrying deletions located between aa 459 and 485. These mutant polypeptides all failed to bind DNA (Fig. 4B, lanes 3, 9, 11, and 12), implicating amino acids in this region in DNA recognition. Further, an adjacent deletion, Δ489-93, bound only as the heterodimer form and not as a homodimer (Fig. 4B, lane 6), suggesting that these residues might contribute to the stability of the DNA bound complex. A similar result was obtained with the point mutation 485D (Fig. 4C, lane 11). Other point mutations within this region bound as both homodimers and heterodimers (Fig. 4B, lane 10, and 4C, lanes 5, 6, and 12). The results of these binding assays are summarized in Table 1. They indicate that an essential DNA binding domain of EBNA-1 lies upstream of the dimerization domains and is located between aa 459 and 487.

Differential binding to mutant oligonucleotides suggests that aa 467, 468, and 477 may participate in DNA recognition. Previously, we had analyzed the precise sequence requirements for EBNA-1 binding using a set of 30-bp oligonucleotides that contained symmetric point mutations in both halves of the palindromic binding site (2). This study revealed that positions 9 and 10 in the consensus half-palindromic sequence did not contribute to EBNA-1 binding and that considerable flexibility could be tolerated at positions 1 and 2. Mutations in positions 3 through 8 of the recognition site had the most deleterious effect on binding. The EBNA-1 deletion constructions identified a DNA binding domain located between aa 459 and 487. To gain further insight into the portion of this domain involved in DNA recognition, we examined the ability of the different EBNA-1 constructions to recognize the previously characterized mutant oligonucleotide probes.

Firstly, 10 of the EBNA-1 mutants that were dimerization competent were individually in vitro translated. The translation products were analyzed on a denaturing polyacrylamide gel (data not shown), and the volumes were adjusted such that equal amounts of ^{35}S-labelled protein were used in the subsequent mobility shift assays. Binding of these mutants to the consensus wild-type oligomer 20 was examined in a mobility shift assay (Fig. 5). (The sequence of oligomer 20 is shown in Fig. 5A, with both copies of the 10-bp consensus half-site boxed.) As was seen in the heterodimerization binding assay (Fig. 4), there is a group of mutants that

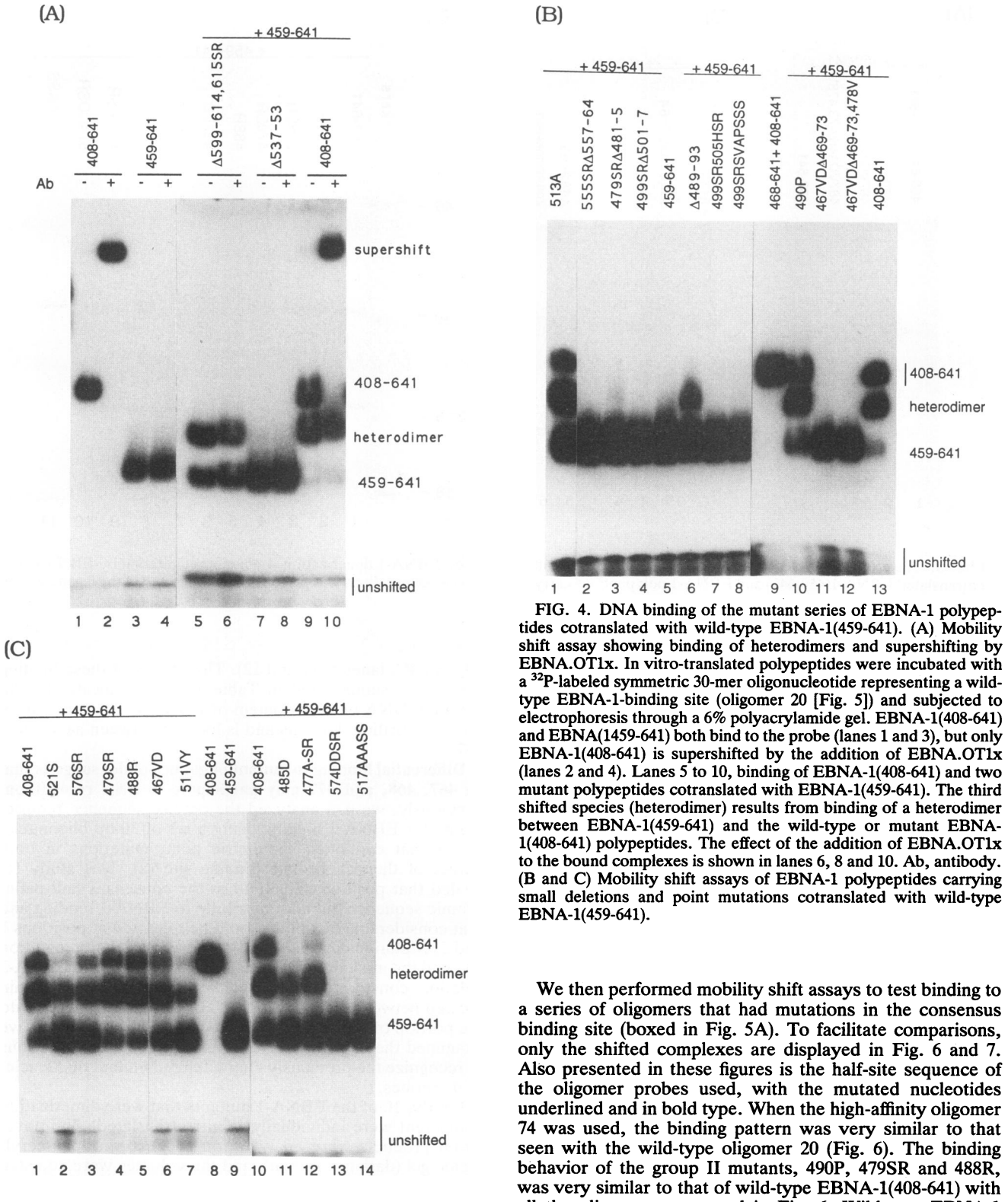

FIG. 4. DNA binding of the mutant series of EBNA-1 polypeptides cotranslated with wild-type EBNA-1(459-641). (A) Mobility shift assay showing binding of heterodimers and supershifting by EBNA.OT1x. In vitro-translated polypeptides were incubated with a ^{32}P-labeled symmetric 30-mer oligonucleotide representing a wild-type EBNA-1-binding site (oligomer 20 [Fig. 5]) and subjected to electrophoresis through a 6% polyacrylamide gel. EBNA-1(408-641) and EBNA(1459-641) both bind to the probe (lanes 1 and 3), but only EBNA-1(408-641) is supershifted by the addition of EBNA.OT1x (lanes 2 and 4). Lanes 5 to 10, binding of EBNA-1(408-641) and two mutant polypeptides cotranslated with EBNA-1(459-641). The third shifted species (heterodimer) results from binding of a heterodimer between EBNA-1(459-641) and the wild-type or mutant EBNA-1(408-641) polypeptides. The effect of the addition of EBNA.OT1x to the bound complexes is shown in lanes 6, 8 and 10. Ab, antibody. (B and C) Mobility shift assays of EBNA-1 polypeptides carrying small deletions and point mutations cotranslated with wild-type EBNA-1(459-641).

cannot bind to the EBNA-1 target sequence. This group, group I, includes Δ537-53, 479SRΔ481-5, and 467VDΔ469-73. All other EBNA-1 mutants bound oligomer 20 with an affinity similar to that of the wild-type EBNA-1(408-641).

We then performed mobility shift assays to test binding to a series of oligomers that had mutations in the consensus binding site (boxed in Fig. 5A). To facilitate comparisons, only the shifted complexes are displayed in Fig. 6 and 7. Also presented in these figures is the half-site sequence of the oligomer probes used, with the mutated nucleotides underlined and in bold type. When the high-affinity oligomer 74 was used, the binding pattern was very similar to that seen with the wild-type oligomer 20 (Fig. 6). The binding behavior of the group II mutants, 490P, 479SR and 488R, was very similar to that of wild-type EBNA-1(408-641) with all the oligomers presented in Fig. 6. Wild-type EBNA-1 binds oligomers 33, 104, 96, and 71 with an intermediate affinity (2). However, several of the mutant polypeptides were completely unable to bind to these oligomer probes (Fig. 6). This group, group III, includes Δ489-93, 485D, and 511VY. In contrast, 477A-SR was able to discriminate

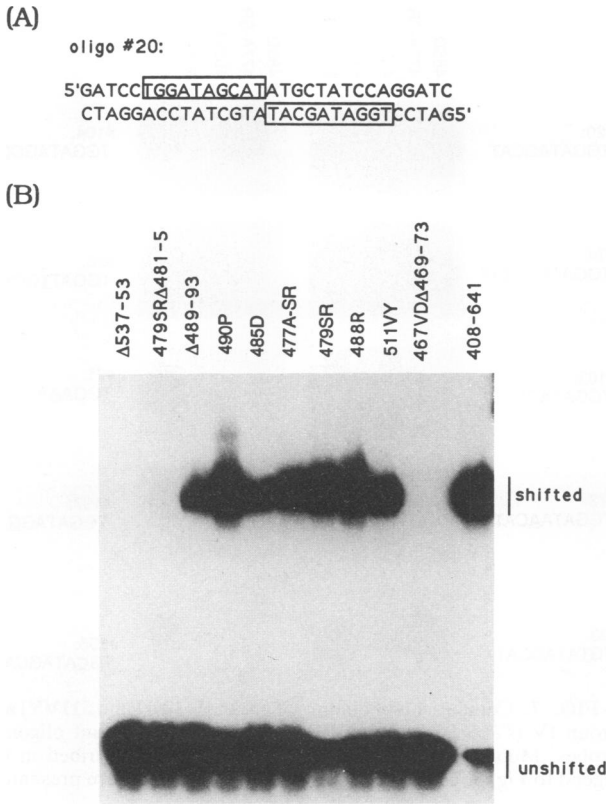

FIG. 5. Comparison of the DNA-binding ability of individually translated wild-type and mutant EBNA-1 polypeptides. (A) Sequence of the oligomer 20 probe. The symmetric core EBNA-1-binding site is boxed. (B) Mobility shift assays using oligomer 20 as the probe.

TABLE 1. Summary of dimerization and DNA-binding characteristics of EBNA-1 polypeptides[a]

EBNA-1 polypeptide	Dimerization	DNA binding	
		Consensus site[b]	Recognition characteristic
408-641	+	+	Wild type
468-641	+	−	
467VD	+	+	Site discrimination
467VDΔ469-73	+	−	
467VDΔ469-73,478V	+	−	
479SR	+	+	Wild-type pattern
477A-SR	+	+	Site discrimination
479SRΔ481-5	+	−	
479SRΔ481-520	−	−	
485D	+	+	High-affinity sites
488R	+	+	Wild-type pattern
Δ489-93	+	+	High-affinity sites
490P	+	+	Wild-type pattern
499SR	+	+	
499SRΔ501-520	−	−	
499SRΔ501-7	−	−	
499SR505HSR	−	−	
499SRSVAPSSS	−	−	
511VY	+	+	High-affinity sites
513A	+	+	Wild-type pattern
517AAASS	−	−	
521S	+	+	
Δ525-32	−	−	
Δ537-53	+	−	
555SR	+	+	
555SRΔ557-64	−	−	
555SRΔ557-75	−	−	
576SR	+	+	
574DDSR	−	−	
576SRΔ578-616	−	−	
Δ599-614,615SR	+	+	Heterodimer binds

[a] Data from Fig. 1 and 3 to 6.
[b] Binding to oligomer 20.

among the intermediate-affinity oligomer probes. It bound oligomers 33 and 104 equally as well as the group II mutants but bound 96 more weakly and failed to bind oligomer 71. Surprisingly, 477A-SR appeared to show weak binding to oligomer 36, which was not bound under these conditions even by the wild-type protein. We classified 477A-SR as a group IV mutant. The mutation in 477A-SR that is contributing to its binding phenotype is clearly the lysine-to-alanine change at position 477. Binding of the protein carrying only the SR mutation, 479SR, is shown alongside 477A-SR in Fig. 6, and its binding properties are indistinguishable from those of wild-type EBNA-1(408-641).

We next compared the binding pattern of another DNA binding domain point mutant, 467VD, with that of two of the group III mutants and the group IV mutant 477A-SR (Fig. 7). The group III mutants, 485D and 511VY, again bound to the high-affinity oligomers 20 and 74, showed reduced binding to the high-intermediate-affinity oligomer 102, and did not bind to any of the other oligomers. Interestingly, the mutant 467VD, like 477A-SR, discriminated among the different intermediate-affinity oligomers. It bound strongly to oligomers 73 and 96 and weakly to oligomer 33, 71, and 197, and it did not bind to oligomer 104. Although 477A-SR and 467VD shared this group IV binding behavior, their binding affinities for the individual mutant oligomers were strikingly different. Mutant 467VD bound to oligomer 96 much more strongly than did 477A-SR and was able to bind to oligomer 71, which was not bound by 477A-SR. On the other hand, 477A-SR bound strongly to oligomers 33 and 104, which were bound only weakly or not at all by 467VD. A summary of the different binding patterns exhibited by the dimerization-positive EBNA-1 mutants is given in Table 2.

The mobility shift assays using individually translated mutant EBNA-1 polypeptides and mutant oligomer-binding sites provided important additional evidence to support the conclusion that the DNA domain of EBNA-1 is located between aa 459 and 487. Furthermore, the unique binding patterns demonstrated by 467VD and 477A-SR strongly implicate aa 467, 468, and 477 as being directly involved in DNA recognition.

DISCUSSION

EBNA-1 is essential for replication of the latent episomal form of the Epstein-Barr virus genome and is one of several proteins involved in the regulation of transcription from the latency C and F promoters. Further, EBNA-1 is the only viral protein expressed in the tumor cells of Epstein-Barr virus-positive Burkitt's lymphoma (27). This observation raises the possibility that EBNA-1 may contribute to the tumorigenic phenotype of these cells. The exact mechanism by which EBNA-1 performs any of its biological functions is incompletely understood. EBNA-1 does not possess any of the enzymatic activities associated with replication proteins such as simian virus 40 T antigen (1, 7, 20), and a specific

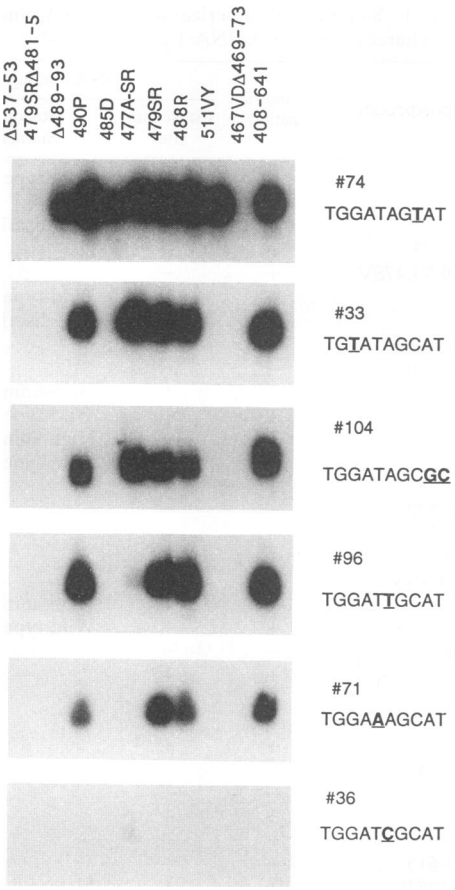

FIG. 6. Differential binding of individually translated wild-type and mutant EBNA-1 polypeptides to oligomer-binding sites carrying symmetric base substitutions. DNA binding was assessed by mobility shift assays. To facilitate comparison, only the upper, shifted DNA bands are presented. The core sequences of the mutant 30-mer probes are shown, with the altered bases in bold type and underlined. The oligomer numbers are those used in a previous study (2). An equal amount of EBNA-1 protein was used in each binding reaction as judged by SDS-PAGE analysis of the radiolabeled polypeptides.

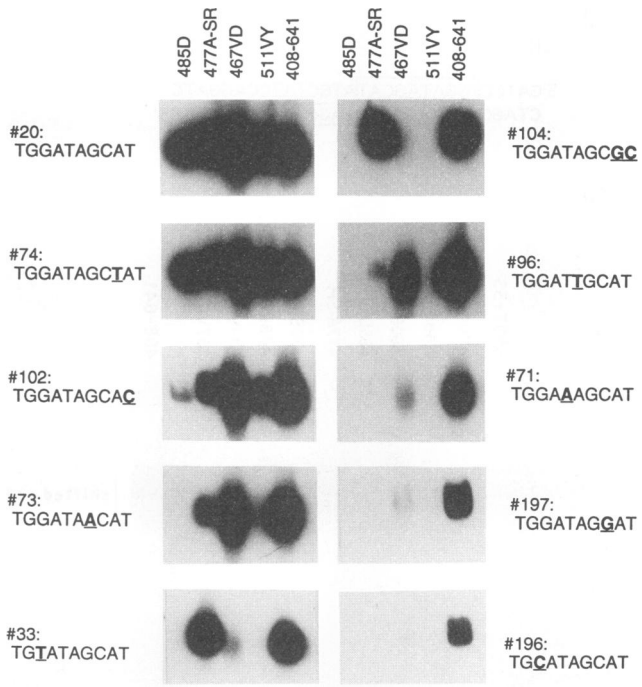

FIG. 7. Comparison of binding of group II (485D and 511VY) and group IV (477A-SR and 467VD) polypeptides to mutant oligomer probes. Mobility shift assays were performed as described in the legend to Fig. 6. Only the upper, shifted DNA bands are presented.

transcriptional activation domain has not been documented. One property that is clearly an integral part of both its replication and transcriptional regulatory functions is the ability to bind specifically to consensus sequences within *oriP* and at the Q locus downstream of the latency F promoter (2, 15, 24). Proteins lacking an intact DNA binding domain are incapable of supporting either transactivation or replication functions (22, 40).

EBNA-1 binds to a 16-bp palindromic recognition sequence as a dimer (1, 8). An earlier analysis of an amino- and carboxy-terminal deletion series located both DNA binding and dimerization functions within the region between aa 459 and 607 (1). The availability of the EBNA.OT1x monoclonal antibody and the determination of its epitope location provided us with an opportunity to use heterodimerization-immunoprecipitation as a primary assay to examine dimerization ability. Confirmatory evidence for dimerization was also obtained in the electrophoretic mobility shift assays using cotranslated EBNA(459-641) and the constructions spanning aa 408 to 641 (Fig. 4). The presence of intermediate mobility complexes in these assays is indicative of heterodimer formation. For those mutants that were DNA-binding negative, dimerization was confirmed by glutaraldehyde cross-linking (data not shown). When applied to a series of mutant EBNA-1 polypeptides, these approaches led to the identification of two segments, aa 501 to 532 and aa 554 to 598, that contained motifs required for dimerization. Mobility shift assays performed with this same series of EBNA-1 mutants identified components of a DNA binding domain between aa 459 and 487, upstream of the dimerization domains. The relative positioning and structures of these domains do not obviously conform to those of any previously defined families of DNA-binding proteins, such as the helix-turn-helix, helix-loop-helix, basic, coiled:coil, or zinc finger proteins (3, 4, 10, 11, 16, 17, 19).

The results of previous protease digestion experiments showed that the entire segment spanning aa 459 to 607 of the polypeptide adopts a highly ordered, protease-resistant structure when EBNA-1 is bound to DNA (32). This observation is relevant to an understanding of the data obtained with the mutant EBNA-1 polypeptides because the properties of a number of them may result from the introduction of constraints on polypeptide structure. For example, mutations in the region from either aa 501 to 532 or aa 554 to 598 destroyed dimerization. Both domains may directly participate in dimerization, or one of these regions may be required to establish a conformation that allows the dimerization motifs located in the other domain to interact. The glycine-proline-rich segment between aa 537 and 553 was specifically susceptible to cleavage with high levels of protease (32), suggesting that this segment of the DNA-bound protein may be exposed or have a less-ordered conformation. The Δ537-553 variant was not capable of homodimerization in cross-

TABLE 2. Relative binding of dimerization-positive EBNA-1 polypeptides to mutant oligomers

EBNA-1 polypeptide	Binding to oligomer no.[a]										
	20	74	102	73	96	33	104	71	197	196	36
Wild type EBNA-1(408-641)	++++	++++	++++	++++	+++	+++	+++	+++	++	+	−
Group I											
467VDΔ469-73	−	−			−	−	−	−			−
479SRΔ481-5	−	−			−	−	−	−			−
Δ537-53	−	−			−	−	−	−			−
Group II											
479SR	++++	++++			+++	+++	+++	+++			−
488R	++++	++++			+++	+++	+++	+++			−
490P	++++	++++			+++	+++	+++	+++			−
Group III											
485D	+++	+++	+	−	−	−	−	−	−	−	−
Δ489-93	+++	+++			−	−	−	−			−
511VY	+++	+++	++	−	−	−	−	−	−	−	−
Group IV											
467VD	++++	++++	++++	++++	+++	+	−	+	+/2	−	−
477A-SR	++++	++++	++	++	+	+++	+++	−	−	−	+/2

[a] ++++, +++, ++, +, and +/2 indicate relative (high to low) levels of EBNA-1 binding; − indicates no binding; combinations showing neither + nor − symbols were not tested.

linking experiments (32) but was able to dimerize with a wild-type aa-408-to-641 polypeptide in both coprecipitation assays (Fig. 1) and cross-linking experiments (data not shown). This deletion variant was the single example of a dimerization-positive, DNA-binding-negative mutant that mapped outside of the region of EBNA-1 from aa 459 to 487. In view of its protease susceptibility, it seems unlikely that the aa 537 to 553 region contributes directly to DNA binding. The lack of DNA-binding ability may be a reflection of the destabilization in dimerization function that is apparent from the inability of this polypeptide to form homodimers. Precedence for such a phenotype comes from studies of E47 in which a variant that could form heterodimers but not homodimers also lacked DNA-binding ability in the heterodimer form (37). Dimerization structures involving both α-helical (6, 10, 23, 30) and β-sheet (12) interactions have been described. There are predicted β-sheet structures within both of the EBNA-1 dimerization segments and a predicted α helix in the second aa 554-598 region (Fig. 8).

The loss of DNA binding demonstrated by three of the deletion mutants served to locate a DNA recognition domain

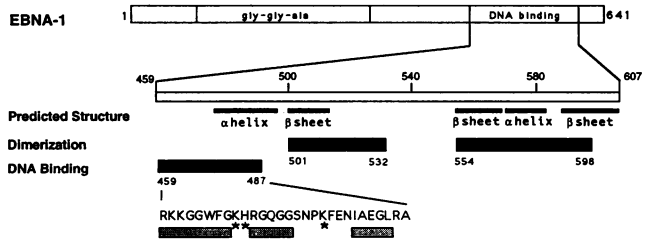

FIG. 8. Location of the dimerization and DNA-binding domains of EBNA-1. The predicted secondary structure (5) of the region spanning aa 459 to 607 is shown. The amino acid sequence of the DNA recognition domain is also provided along with the positions of the deletions that abolish DNA binding (stippled bars) and the substitutions that affect DNA recognition (*).

between aa 459 and 487 (Fig. 8). Further, two of the group III mutants were located adjacent to the right-hand boundary of this domain. The leucine-to-aspartate change at aa 485 and the aa 489 to 493 deletion bound only as heterodimers when cotranslated with wild-type EBNA-1(459-641), and although they bound normally to wild-type probe when individually translated, they failed to bind to any of the intermediate-affinity mutant probes. The loss of binding to these latter probes was unrelated to the position of the mutated nucleotide within the binding site and hence is unlikely to involve direct DNA contacts. Leucine 485 and aa 489 to 493 may be contributing to a conformation that favors DNA interactions with the recognition amino acids. Protease digestion experiments indicated that the region spanning aa 459-607 undergoes a conformational change on binding (32). This structural rearrangement may be essential to maximize contacts between the polypeptide and DNA interfaces. If the group III mutations interfere with the ability to make this conformational change, then the DNA contacts made by these polypeptides might be sufficiently destabilized that binding could occur only to high-affinity-binding sites. The other group III mutant, 511VY, lies 18 aa downstream and converts two glycine residues to hydrophobic valine and tyrosine residues. It should be noted that polypeptides containing other nonconservative amino acid mutations in the same vicinity, namely leucine 488 to arginine and alanine 490 to proline bind the mutant oligomers indistinguishably from the wild-type polypeptide.

The behavior of the group IV mutants is also particularly interesting because it implies that these particular amino acids may be involved directly in DNA recognition. Conversion of aa 467 and 468 from lysine and histidine to valine and aspartate (467VD) and aa 477 from lysine to alanine (477A-SR) resulted in EBNA-1 polypeptides that were able to discriminate between different intermediate-affinity DNA probes. A comparison of relative binding showed that while 467VD bound to the majority of the mutant oligomers with a higher affinity than 477A-SR (Fig. 7; Table 2), there were two

notable exceptions; oligomers 33 and 104, which have mutations at position 8 and at positions 1 and 2, respectively. An examination of the binding of 477A-SR (Fig. 6; Table 2) showed that this polypeptide, which bound as well as the wild-type protein to oligomers 33 and 104, bound only weakly to oligomer 96 and did not bind at all to oligomer 71. Oligomer 96 carries a nucleotide change at position 5 of the binding site, and oligomer 71 is altered at position 6. Furthermore, the aa-477 mutant was able to bind, albeit weakly, to an oligomer (no. 36) carrying a mutation at position 5 that was not bound by the wild-type protein. EBNA-1-binding site recognition is most likely mediated by a combination of contacts with bases in the major groove (2, 18) and contacts with the phosphate backbone. The contribution to recognition of the lysine and histidine at aa 467 and 468 and the lysine at aa 477 can only be speculated upon at this time. The most deleterious changes for binding of 467VD were located at disparate positions in the binding site, positions 1, 2, and 8, and the interactions involving these amino acids would appear to be complex. Because the mutations that most strongly affected binding of 477A-SR were each located at nucleotide 5 or 6 of the binding site, the contacts made by lysine-477 may therefore involve these positions.

A large number of DNA-binding proteins utilize a DNA recognition motif that is an alpha helix. The E2 transcriptional regulator of papillomavirus and the GCN4 bZIP protein contain such a motif (6, 12) as do the helix-turn-helix proteins. Examples of this latter group include many of the prokaryotic transcriptional regulatory proteins and the eukaryotic homeodomain proteins (reviewed in references 8 and 11). On the other hand, both the yeast *met* repressor and the TFIID TATA-box binding protein utilize anti-parallel β strands or β ribbons as the DNA recognition motif (21, 34). The EBNA-1 DNA binding domain identified in this study overlaps with a predicted α helix, but the exact nature of the EBNA-1 DNA recognition motif remains to be elucidated.

The bHLH group of proteins contain a basic DNA recognition domain and an adjacent dimerization domain consisting of two amphipathic alpha helices separated by a variable loop that is 9 to 13 aa in length (4, 10, 37). Comparison of proteins within this group has identified a consensus sequence for the bHLH motif (4). Using computer analysis, Inoue et al. (13) matched this consensus to a region of EBNA-1 between aa 465 and 587 and suggested on the basis of this amino acid alignment that EBNA-1 might belong to the bHLH class. The degree of match to the bHLH consensus was intriguing but imperfect. The proposed basic region of EBNA-1 contained a three of six match to the bHLH consensus, and the matches for helix 1 and helix 2 were three of six and five of eight, respectively, while the loop region would contain an extended 83 aa. The behavior of a number of the EBNA-1 variants does not appear to be compatible with the bHLH model. This is particularly true of the EBNA-1 mutants in the region (aa 477 to 493) of predicted helix 1. Deletion of five aa (aa 481 to 485) from the center of the helix removes two of the consensus hydrophobic residues and destroys the 4:3:4 hydrophobic pattern which would create the hydrophobic interface between the predicted alpha helices. Nonetheless, the Δ481-485 mutant was dimerization competent. We converted two of the consensus hydrophobic residues to charged residues (Leu-485 to Asp and Leu-488 to Arg). Mutation of the equivalent residues in the bHLH protein E47 from hydrophobic to charged abolished dimerization of E47 (37), but these mutations had no effect on dimerization of EBNA-1. We also introduced a helix-breaking proline residue into the predicted helix 1 at aa 490 and found that this mutation had no effect on dimerization. Further, mutations in the loop region of a bHLH protein have been shown not to affect dimerization (37), whereas two separate grouped mutations (499SR505HSR and 517AAASS) in the predicted loop of EBNA-1 each abolished dimerization. The DNA recognition region mapped in our experiments, aa 459 to 487, does in fact overlap with the basic domain predicted by the sequence comparison, and the second dimerization domain, aa 554 to 598, overlaps with the predicted helix 2. However, the experimental data is not compatible with a dimerization domain being located in the region of predicted helix 1. Since the spacing between the basic DNA-binding domain and the first helix of the dimerization domain is constant in bHLH proteins, this finding would argue against the inclusion of EBNA-1 within this protein family. The partial amino acid match to the bHLH consensus within the EBNA-1 DNA recognition domain and the second dimerization domain may represent convergent evolutionary selection of particular amino acid combinations that favor the type of interactions involved in DNA binding and protein-protein contacts.

ACKNOWLEDGMENTS

We thank Mabel Chiu and J.-C. Zong for excellent technical assistance and Sarah Heaggans for manuscript preparation.

This work was supported by Public Health Service grants RO1 CA42245 and RO1 AI31454 from the National Institutes of Health.

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