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The DNA-Binding Properties of the Major Regulatory Protein α4 of Herpes Simplex Viruses

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The transition from the expression of α, the first set of five herpes simplex virus genes expressed after infection, to β and γ genes, expressed later in infection, requires the participation of infected cell protein 4 (α4), the major viral regulatory protein. The α4 protein is present in complexes formed by proteins extracted from infected cells and viral DNA fragments derived from promoter domains. This report shows that the α4 protein forms specific complexes with DNA fragments derived from 5′ transcribed noncoding domains of late (γ_2) genes whose expression requires viral DNA synthesis as well as functional α4 protein. Some of the DNA fragments to which α4 binds do not contain homologs of the previously reported DNA binding site consensus sequence, suggesting that α4 may recognize and interact with more than one type of DNA binding site. The α4 proteins can bind to DNA directly. A posttranslationally modified form of the α4 protein designated α4c differs from the α4a and α4b forms with respect to its affinity for DNA fragments differing in the nucleotide sequences of the binding sites.

THE HERPES SIMPLEX VIRUS 1 AND 2 (HSV-1 and HSV-2) genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (*1*). The α genes are expressed first, and functional α proteins and especially the major regulatory protein, infected cell protein 4 (ICP4) or α4, are required for the expression of the β and γ genes later in infection (*2–6*). Previous studies have also suggested that the α4 protein may negatively regulate α gene expression (*3, 6–8*). The mechanism by which α4 protein positively regulates some genes and negatively regulates others is not known. The α4 protein in crude cellular extracts binds to DNA (*9*) and forms complexes that decrease the electrophoretic mobility in nondenaturing gels of specific DNA fragments from promoter domains of HSV genes (*10*). Monoclonal antibodies to the α4 protein further decrease the electrophoretic mobility of these complexes (*10*), and deoxyribonuclease (DNase) I footprints of some binding sites have suggested a DNA-binding consensus sequence (*10, 11*). In the studies described here on the interaction of the α4 proteins with DNA fragments derived from α genes and from late, γ_2 genes whose expression requires viral DNA synthesis as well as functional α proteins, we used three sets of fragments. The first was derived from the $\gamma_2$42 gene specifying ICP42, a γ_2 protein (*12, 13*), and consisted of 11 fragments designated D′1 to D′11 spanning the region of −179 to +104 relative to the transcription initiation site at +1 (Fig. 1, A and B). The second set contained a fragment extending from nucleotide +8 to nucleotide +194 relative to the transcription initiation site at +1 of the gene specifying the α-trans-inducing factor (αTIF), the infected cell protein 25 (ICP25), which is packaged in the virion and induces α genes after infection (*14–16*). The third set consisted of two DNA fragments from the α4 gene, which was previously shown to contain two α4 protein binding sites (*10, 17*). Fragment α4-2 spans the transcription initiation site, whereas fragment α4-1 is located between −140 and −199 relative to the transcription initiation site (*18*). The DNA fragments were terminally labeled with ^{32}P and mixed with mock-infected or infected cell nuclear extract in the presence of excess synthetic competitor DNA [poly(dI)·poly(dC)], and the reaction mixture was electrophoretically separated in a nondenaturing gel. To demonstrate the presence of α4 protein in the labeled DNA–protein complex, the binding of proteins in the nuclear extract with the labeled DNA was also done in the presence of monoclonal antibody H640 to α4 protein. The results were as follows.

1) The location and the number of α4 protein binding sites vary from one gene to another. The major and readily detected binding site in the αTIF gene is located in the 5′ transcribed noncoding leader sequence (Fig. 2, lanes 37 to 39). In the $\gamma_2$42 gene (Fig. 2), we detected at least four binding sites, two in the promoter domain and two in the leader sequence. The first binding site is in fragment D′1. The position of the second binding site is defined by fragment D′11, which binds, and fragment D′10, which does not, though the complete binding domain may extend into D′10. The location of the third site is unambiguously defined by D′8 and D′9, which bind, and D′10, which does not. The fourth site is contained in D′6, which binds; failure of D′7 to bind may be due to the Rsa I cleavage of the binding site. DNase I protection studies on the D′5 fragment, which contains the third and fourth sites, do not support the hypothesis that the third and fourth binding sites are components of a single extended binding site (*19*). The decrease in the mobility of the DNA-protein complexes caused by the monoclonal antibody to α4 confirmed the presence of the α4 protein in the complexes obtained with these fragments (Fig. 2).

2) In instances where the α4 protein–DNA probe formed multiple bands, all of the bands were shifted to a slower migrating position by the monoclonal antibody to α4. The same fragment formed both single and double bands (Fig. 2, lanes 2, 3, 5, and 6). Whereas fragment D′2 formed a strong, slow migrating band and a weak, fast migrating band, the two bands formed by the slightly larger D′3 fragment were of equal intensity. For a given nuclear extract and reagent concentration, the results were reproducible.

3) Faber and Wilcox (*11*) derived the consensus sequence ATCGTCnnnnYCGRC for the DNA binding site of the α4 protein on the basis of analyses of one binding site of this protein to the promoter domain of the HSV-1 glycoprotein D gene and two binding sites in the pBR322 plasmid DNA. Homologs of this sequence are present in the promoter domain of the α0 gene (ATCGTCactgCCGcC) (*10*). In our assays, homologs were present in the αTIF gene

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downstream from nucleotide +154 (ATCGTCtttcCCGta) and in the D'6 DNA fragment containing the fourth binding site (ATCGTaccccCaaGC), but not in the other DNA fragments from the $\gamma_2$42 gene that bound the α4 protein or in the α4-1 DNA fragment reported earlier (*10*). These results suggest that the α4 protein may recognize and interact with more than one type of DNA sequence.

4) We observed an additional virus-specific complex formed by a D'1 fragment in the presence of infected cell extracts. The mobility of this complex was not altered by the monoclonal antibody to α4 (Fig. 2, lanes 2 and 3).

The observation that the α4 protein was present in DNA-protein complexes forming multiple bands and containing DNA sequences differing from the published consensus sequence for the DNA-binding sites raised the question whether α4 protein binds to DNA directly or requires other proteins for binding to DNA. Other investigators reported that partially purified α4 protein bound DNA only after reconstitution with extracts of uninfected cells (*9*). We therefore electrophoretically separated in denaturing gels the proteins contained in nuclear extracts of cells harvested 24 hours after infection with HSV-1 strain F [HSV-1(F)]. The proteins were transferred to a nitrocellulose sheet, renatured, and reacted with ^{32}P-labeled α0 or D'1 HSV-1 DNA fragments (Fig. 1) or with a labeled 181-bp fragment from pUC19 in the presence or absence of a 50-fold excess of unlabeled 181-pUC19 DNA. The nitrocellulose sheets were then exposed to x-ray film for autoradiography and reacted first with the monoclonal antibody to α4 and then with antibody to mouse immunoglobulin G (IgG) conjugated to peroxidase. The results (Fig. 3, A and B) indicated that the probes bound several proteins in the nuclear extracts of infected cells. However, only the α0 and the D'1 probe bound the α4 protein band. Moreover, the binding of the labeled α0 probe to the α4 protein appeared to be stronger than that of the labeled D'1 probe, and neither appeared to be affected by the competitor DNA.

The α4 proteins form at least three bands designated α4a, α4b, and α4c on electrophoresis in denaturing polyacrylamide gels (*20*). The newly synthesized protein comigrates with the α4a form, and the slower mobility of the α4b and α4c forms reflects posttranslational modifications of the proteins (*20–23*). To achieve a better separation of the various α4 forms for analyses of their affinity to DNA fragments containing the α4 binding sites, we separated a nuclear extract of HeLa cells infected with HSV-2 strain G [HSV-2(G)] by electrophoresis on a denaturing polyacrylamide gel. The proteins were transferred to a nitrocellulose sheet that was cut into strips. Duplicate strips were incubated with different amounts of labeled probes and then reacted with the monoclonal antibody to the α4 protein. All strips were subjected to identical autoradiography, photographed, and printed under identical conditions. The results were as follows (Fig. 3): The affinity of the labeled γ_2 probes (D'1 and D'5) was lower than that of the α0 probe for the immobilized α4 proteins. Moreover, the affinity of the γ_2 probes and particularly D'1 for the α4c protein was lower than that of the α0 probe. This is clearly evident from comparisons of the distribution of labeled probe DNA on the α4 proteins in lanes 2 and 4 showing the binding of 3×10^5 cpm of the α0 probe and 10^6 cpm of the D'1 probe. The α4-2 probe spanning the transcription initiation site of the α4 gene has a higher affinity for the α4 proteins than the α4-1 probe.

These results show that unlike αTIF, which requires the participation of a host protein for binding to its cis site (*24, 25*), the α4 protein can bind directly to viral DNA. There also appears to be more than one type of α4 DNA binding site. In the HSV-1 genome, homologs of the previously published consensus sequence were reported in genes specifying glycoprotein D (*11*), α0 (*10*), α4 (α4-2 site) (*17, 26*), and αTIF. The second (α4-1 site) binding site in the α4 gene (*10*) and binding sites 1, 2, and 3 in the $\gamma_2$42 gene do not match the consensus and appear to represent alternative binding sites. In their reactions with the immobilized α4 proteins, the DNA fragments containing a homolog of the consensus sequence ATCGTCnnnnYCGRC (for example, α0 and α4-2) appear to bind more readily to the α4c protein than the DNA fragments with alternative binding sites. These studies

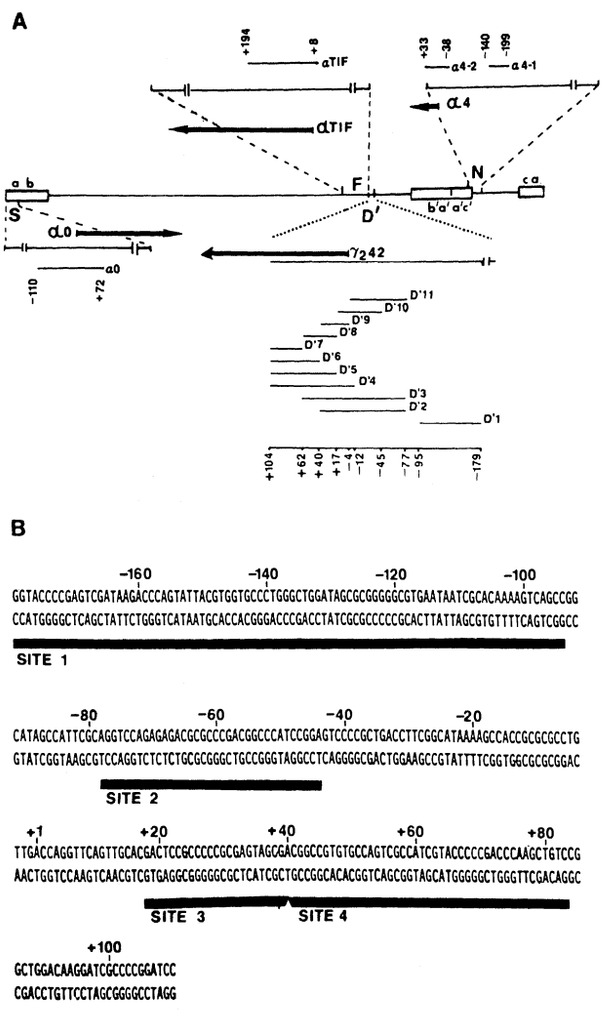

Fig. 1. Size, nucleotide sequence, and genomic locations of HSV-1 DNA probes. (**A**) The center horizontal line represents the HSV-1 genome with boundaries of four Bam HI fragments indicated: S, F, N, and D'. The rectangles represent the inverted repeat sequences ab, b'a'a'c', and ca. Expanded scales show the locations of the α0, α4, $\gamma_2$42, and αTIF gene domains. The arrows show the transcribed domain. The thin lines and numbers in the lower portion indicate the location of the DNA probes and the boundaries of the probes relative to the transcription initiation site at +1 (*12, 13, 16, 17*). The α0, α4-1, and α4-2 probes were described elsewhere (*10*); αTIF is the 186-bp Sal I–Ava I fragment. The sizes and restriction enzyme boundaries of the D'1 to D'11 DNA probes were as follows: D'1, Kpn I–Hpa II, 84 bp; D'2, Ava II–Xma III, 117 bp; D'3, Ava II–Rsa I, 139 bp; D'4, Bss HI–Bam HI, 116 bp; D'5, Hinf I–Bam HI, 87 bp; D'6, Xma III–Bam HI, 64 bp; D'7, Rsa I–Bam HI 42 bp; D'8, Hinf I–Rsa I, 45 bp; D'9, Hinc II–Xma III, 66 bp; D'10, Hinf I–Hinf I, 62 bp; D'11, Ava II–Hinc II, 73 bp. (**B**) The nucleotide sequence of the 5' nontranscribed and that portion of the 5' transcribed noncoding domain of the $\gamma_2$42 gene contained in HSV-1(F) Bam HI D'. The HSV-1(F) domain of this gene sequenced by Sanger's method as described (*35*) differs from the corresponding domain of the HSV-1(KOS) (*13*) with respect to several nucleotides at positions +5 to +9 and +94 to +107. The thick lines indicate the minimal domains of the α4 protein binding sites as determined in this study.

suggest that the α4 proteins may have different regulatory functions and that these are reflected in different DNA-binding sites. Previous studies have suggested that the binding site corresponding to the ATCGTCnnnnYCGRC homolog negatively regulates α gene expression (*8*). If this consensus sequence enables negative regulation, the alternate binding sites may represent cis sites for positive regulation by the α4 proteins. This possibility and the potentially different functions of the α4c protein remain to be explored further.

Lastly, α4 binding sites have been detected in the leader sequence of four γ genes, that of (i) the αTIF gene (Fig. 2), (ii) the $\gamma_2$42 gene (Fig. 2), (iii) the US11 gene, and (iv) the glycoprotein C gene (*27*). Consistent with the expectation that the binding sites represent regulatory signals, the transfer of a sequence from the leader sequence of the $\gamma_2$42 gene to the corresponding region of the thymidine kinase gene, a β gene, conferred upon this gene the characteristics of a γ gene (*28*). The finding of α4 binding sites in the 5′ transcribed noncoding domains of HSV genes has not been previously reported although regulatory transcriptional cis-acting signals in 5′ transcribed noncoding sequences have been reported in other systems (*29–34*).

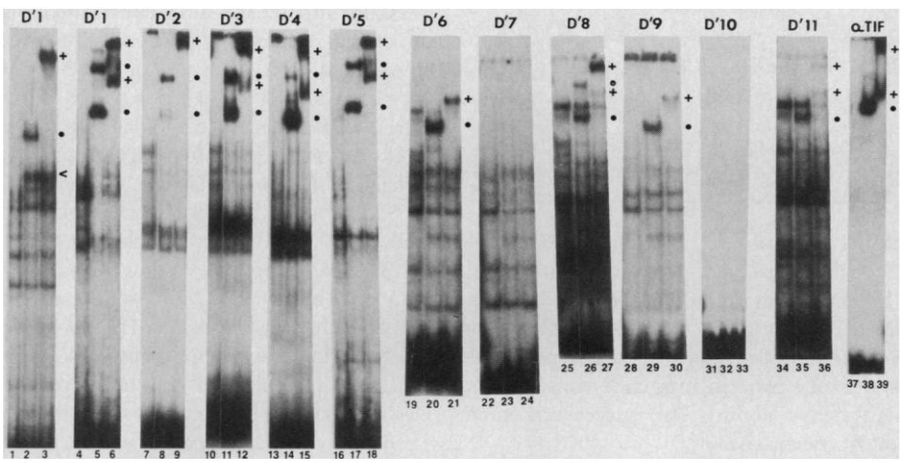

Fig. 2. Autoradiographic visualization of labeled DNA fragments whose electrophoretic mobility in nondenaturing polyacrylamide gels was reduced by formation of complexes with proteins from nuclear extracts. Approximately 2.5 ng of ^{32}P-labeled DNA and 1.7 μg of poly(dI·dC) were reacted with 1 μg of proteins extracted from nuclei of HeLa cells (*10, 36*) for 0.5 hour before electrophoresis on a 4% polyacrylamide gel or with nuclear extract from infected cells. D′1 through D′11 are ^{32}P-labeled DNA probes from the $\gamma_2$42 gene; their location in the Bam HI D′ fragment is shown in Fig. 1A. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, and 37: mock-infected cell nuclear extract; lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, and 38: HSV-1 (24-hour) or HSV-2 (12-hour) infected cell nuclear extract; lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, and 39: 1 μg of monoclonal antibody H640 to the α4 protein was added to the mixture concurrently with the other reactants (*10*). Filled circles identify the DNA-infected cell protein complexes whose electrophoretic mobility was reduced by antibody to the α4 protein as shown in the adjacent lane and indicated by a +. < identifies a DNA-protein complex that contains an infected cell-specific protein that did not react with the monoclonal antibodies to α4.

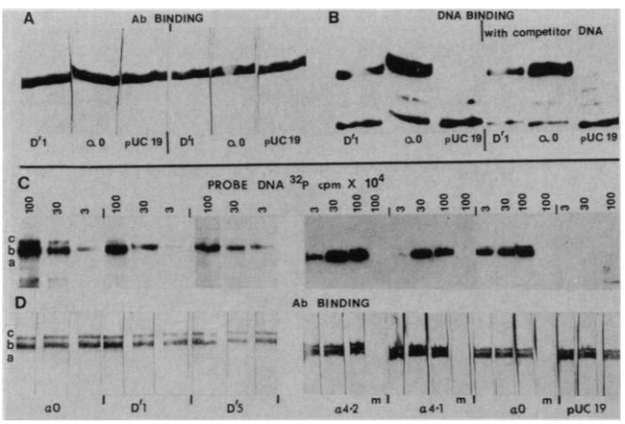

Fig. 3. Specificity of DNA fragment binding to immobilized, renatured HSV-1–infected HeLa cell nuclear proteins. Nuclear proteins (*10, 24*) extracted from HeLa cells 24 hours after infection with HSV-1 or 14 hours after infection with HSV-2 were electrophoretically fractionated on a preparative SDS–9.3% polyacrylamide gel and electrically transferred to a nitrocellulose sheet. Immediately after transfer, the nitrocellulose was gently washed through three changes of buffer A [10 m*M* tris-HCl, (*p*H 7.2), 5% skim milk, 10% glycerol, 2.5% Nonidet P40, 0.1 m*M* dithiothreitol, and 150 m*M* NaCl)] for 3 hours. The nitrocellulose was cut into strips, briefly rinsed in buffer B [10 m*M* tris-HCl (*p*H 7.2), 50 m*M* NaCl, and 0.125% skim milk] and placed in a hybridization bag containing 2 ml of buffer B and ^{32}P-labeled probe DNA (Fig. 1). After incubation overnight at room temperature, the strips were removed from the bag, washed through three changes of buffer B without skim milk, air dried, mounted, and exposed to x-ray film. (**B**) Autoradiogram of HSV-1 proteins reacted with 10^5 cpm of indicated ^{32}P-labeled probes in the absence of competitor DNA or in the presence of unlabeled 181-bp Pvu II–Hind III pUC19 DNA. (**A**) Photograph of the strips in (B) after reaction with antibody H640 to the α4 protein. (**C**) Autoradiogram of HSV-2 proteins reacted with the indicated amounts of the respective labeled DNA probe. Labels a, b, and c indicate the three forms of the α4 protein differentiated by one-dimensional electrophoretic separation. (**D**) Photograph of the strips in (C) after reaction with antibody H640 to the α4 protein. Strips marked "m" contain mock-infected nuclear proteins.

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Reshaping Human Antibodies: Grafting an Antilysozyme Activity

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The production of therapeutic human monoclonal antibodies by hybridoma technology has proved difficult, and this has prompted the "humanizing" of mouse monoclonal antibodies by recombinant DNA techniques. It was shown previously that the binding site for a small hapten could be grafted from the heavy-chain variable domain of a mouse antibody to that of a human myeloma protein by transplanting the hypervariable loops. It is now shown that a large binding site for a protein antigen (lysozyme) can also be transplanted from mouse to human heavy chain. The success of such constructions may be facilitated by an induced-fit mechanism.

FOR PASSIVE IMMUNITY OR ANTIBODY therapy in humans, monoclonal antibodies designed to eliminate toxins, viral and bacterial pathogens, or other cells would ideally be of human origin (*1*). Unfortunately it has proved difficult to make human monoclonal antibodies by hybridoma technology (*2*). Chimeric antibodies with mouse variable and human constant domains have been constructed by linking together the genes encoding each domain (*3, 4*), and expressing the recombinant antibodies in myeloma cells. However, the mouse variable region may itself be seen as foreign (*1*). We have therefore attempted to insert the antigen-binding site of a mouse antibody, rather than the whole variable region, directly into a human antibody. In previous work, the three heavy-chain hypervariable regions [or complementarity-determining regions (CDRs)] from a mouse antibody to a hapten were transplanted onto the framework regions of the heavy-chain variable (V_H) domain of a human myeloma protein. In combination with the mouse light chain, the reshaped heavy chain bound tightly to hapten (*5*). Although it seems likely that both heavy and light chains make contacts to the hapten, the relative contribution of each chain is unknown. Nor is it clear whether the small hydrophobic hapten NP (4-hydroxy-3-nitrophenacetylaminocaproate) simply binds to a hydrophobic pocket at the base of the hypervariable loops. By contrast, the three-dimensional structure of the complex of lysozyme and the mouse antibody D1.3 has been solved (*6*), and about 690 Å^2 of the solvent-accessible surface of the antibody is buried on complex formation. Both V_H and light-chain variable (V_L) domains make extensive contacts to lysozyme, but most of the hydrogen-bonding contacts are made to the CDRs of the heavy chain. Thus, of 12 hydrogen bond interactions proposed (*6*), 9 are made to the heavy chain. We have replaced the hypervariable loops of the human NEW heavy chain (*5*) with those from the D1.3 heavy chain.

The variable domains of the mouse antibody to lysozyme were cloned and sequenced as described (*7*). To reshape the NEW heavy chain, we started from a synthetic gene in an M13 vector (Fig. 1b) containing the framework regions of human NEW and the CDRs from mouse antibody B1-8 (*5*). Long oligonucleotides with multiple mismatches with the template (*8*) were used to replace each of the hypervariable loops in turn by site-directed mutagenesis: the central mismatched portion of the primer encoded each CDR of the D1.3 heavy chain, and the 5′ and 3′ ends of the primer were complementary to the flanking framework regions. Thus after three rounds of mutagenesis, the reshaped gene (HuV_H-LYS) encoded the framework regions of NEW with the hypervariable regions of D1.3 (Figs. 1c and 2). This was assembled with the heavy-chain constant region of human immunoglobulin G2 (HuIgG2) (*9*) to give the plasmid pSVgpt-HuV_HLYS-HuIgG2. The plasmid was transfected by electroporation (*10*) into the myeloma line J558L (*11*), which secretes a mouse λ light chain. Transfectants resistant to mycophenolic acid were screened for secretion of immunoglobulin by gel electrophoresis of supernatants from [^{35}S]methionine-labeled cells. The secreted product (HuV_HLYS-HuIgG2, λ) was purified on protein A–Sepharose; the λ light chain was exchanged for the D1.3 κ light chain in vitro; and the HuV_HLYS-HuIgG2, κ antibody was purified (*12*). In parallel experiments as control, the mouse D1.3 variable region (MoV_H-LYS) was attached to the heavy-chain constant region of mouse IgG1 (MoIgG1) in a pSVgpt vector (pSVgpt-MoV_HLYS-MoIgG1), and antibody was expressed and reassociated as above (Fig. 1d).

Fluorescence quench was used to measure

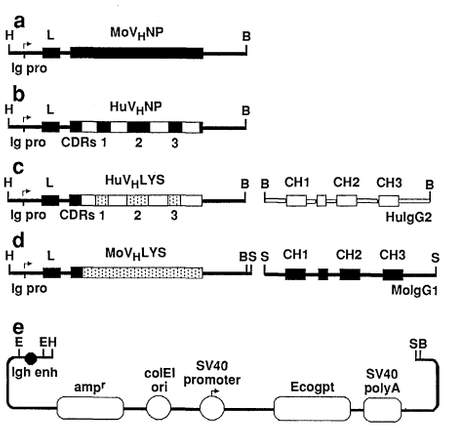

Fig. 1. Vectors for heavy-chain expression. (**a**) Mouse V_{NP} gene (*4*) and designated here as MoV_HNP, (**b**) reshaped HuV_{NP} gene (*5*) designated here as HuV_HNP, (**c**) HuV_HLYS gene with the heavy-chain constant region gene of human IgG2, (**d**) MoV_HLYS gene with the heavy-chain constant region gene of mouse IgG1, and (**e**) the backbone of the pSVgpt vector with immunoglobulin heavy-chain enhancer (Igh enh) (*4*). The V_H domains are denoted in black, stippled, or open boxes to signify sequences encoding mouse antibody to NP (B1-8), mouse antibody to lysozyme (D1.3), and human myeloma protein (NEW), respectively. The constant domains are denoted in black or open boxes to signify sequences encoding mouse or human constant domains. Ig pro, heavy-chain promoter; L, leader exon; H, Hind III; B, Bam HI; S, Sac I; E, Eco RI restriction sites. The reshaped NEW heavy chain was expressed from a vector derived from the pSVgpt-HuV_HNP vector (b). Thus the HuV_HNP gene, cloned initially in M13mp9 as a Hind III–Bam HI fragment, contains heavy-chain promoter, the CDRs of the B1-8 antibody, and the framework regions of human NEW (*5*). The CDRs of B1-8 were then replaced with those of D1.3 using long mutagenic oligonucleotides (see Fig. 2). The Hind III–Bam HI fragment, now carrying the HuV_HLYS gene (c) was excised from the M13 vector and cloned into the pSV vector (e) along with a Bam HI fragment encoding the heavy-chain constant region of human IgG2 (*9*). The construction of the pSVgpt-MoV_HLYS-MoIgG1 vector to express recombinant D1.3 is summarized in (d).

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