Proc. Natl. Acad. Sci. USA
Vol. 78, No. 6, pp. 3403–3407, June 1981
Biochemistry

Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles

(synthetic peptides/vaccines/sequence-specific antibodies)

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Communicated by Floyd E. Bloom, February 26, 1981

ABSTRACT **Thirteen peptides corresponding to amino acid sequences predicted from the nucleotide sequence of the hepatitis B surface antigen were synthesized chemically. The free or carrier-linked synthetic peptides were injected into rabbits, and 7 of the 13 elicited an antipeptide response. Antisera against four of the six soluble peptides longer than 10 amino acids were reactive with native antigen and specifically precipitated the 23,000- and 28,000-dalton forms from Dane particles. As the hepatitis molecule had not been chosen for study because of any structural feature suggesting unique opportunities for success, these results suggest that the strategy is general and should work for any protein as long as enough domains are studied. Peptides such as these could prove to be ideal vaccines.**

The cloning and sequence determination of genes have greatly increased our knowledge of the structure of proteins and suggested mechanisms by which some are synthesized, processed, and transported. As we move to the study of uncharted genetic regions, however, we encounter a gap between the ease with which a gene can be cloned and sequenced and the unequivocal assignment of its protein product. Recently, a solution to this problem was offered that demonstrated that one could produce antibodies to a few chemically synthesized peptides predicted from newly solved nucleotide sequences and then use these antibodies to define the protein product of the gene in question (1–3). The most important feature of antibodies made in this way is that they are directed against a small region of the protein, determined in advance by the investigator, and are thus unique biochemical reagents. Because this technology could have significant implications, it was important to learn whether the somewhat limited experience could be generalized and any "rules" that might be derived concerning which regions of proteins offered the best possibilities for selection of peptides likely to yield useful antibodies. We selected as models two genes whose nucleotide sequences were known and whose protein products were of both theoretical and practical interest. The first was the major envelope protein of the hepatitis B genome, a molecule that, because of its extreme hydrophobicity, offered an interesting challenge to the technology. The second was the hemagglutinin of influenza virus because its complete crystallographic structure is known (4); thus, one could correlate how antibodies to protein domains of known molecular location perturb virus infectivity and, in fact, what the structural correlates of antigenicity are for the molecule. We report here our studies on the hepatitis B surface antigen (HB_SAg).

HB_SAg is a glycosylated protein and the major surface antigen of the 42-nm particles (Dane particle) of hepatitis B virus (5–7). The HB_SAg contains group- and type-specific determinants and is thought to be the major target of neutralizing antibody (6). Purified preparations of HB_SAg are physically heterogenous and consist of at least seven polypeptides ranging in size from 23,000 to 97,000 daltons (6). By mass, the major HB_SAg component has a size of 23,000 daltons (6). Immunological studies have shown that the proteins of different sizes share common determinants, suggesting that the physical polymorphism reflects different degrees of glycosylation and aggregation. The amino acid sequence of the 226 amino acid HB_SAg deduced from the published nucleotide sequences (8–10) is given in Fig. 1. Overall, the HB_SAg is an exceedingly hydrophobic molecule that is rich in proline and cysteine residues. We have studied HB_SAg by the unpublished computer program of J. E. Kyte and R. F. Doolittle, which makes a running average of local hydrophobicity and has been shown to be highly predictive of internal and external residues of proteins whose structures are known. If HB_SAg is considered in terms of domains, one can discern three hydrophobic and two "hydrophilic" areas in the molecule. (For simplicity, we speak of hydrophilic domains, but, in fact, the molecule is so hydrophobic that it is probably more accurate to think in terms of hydrophobic and not-so-hydrophobic domains.) The largest and most hydrophobic region spans approximately positions 80–110. This domain is flanked by two "hydrophilic" domains encompassing positions 45–80 and 110–150. The other two hydrophobic domains are found at the NH_2 and COOH termini. Most of the cysteines are clustered in the two hydrophilic domains. Overall, then, one has the picture of a hydrophobic molecule with potential for complex conformation dictated by frequent bends at prolines and intrachain disulfide bonds at cysteines. Such a structure is consistent with the known resistance of the molecule to denaturation and digestion by proteolytic enzymes (11). Thus, one might have expected that most of the antigenic determinants of this molecule would be formed by amino acids distant in the linear protein sequence but held close together in space by the tertiary structure. As such, HB_SAg is an excellent test case for generalizing the use of continuous amino acid sequences in designing synthetic antigens.

MATERIALS AND METHODS

Synthesis of Peptides. The peptides were synthesized in collaboration with J. K. Chang of Peninsula Laboratories by using the solid-phase methods developed by Merrifield and his colleagues (for review, see ref. 12). Each synthetic peptide was

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Abbreviations: HB_SAg, hepatitis B surface antigen; KLH, keyhole limpet hemocyanin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; P_i/NaCl, phosphate-buffered saline.

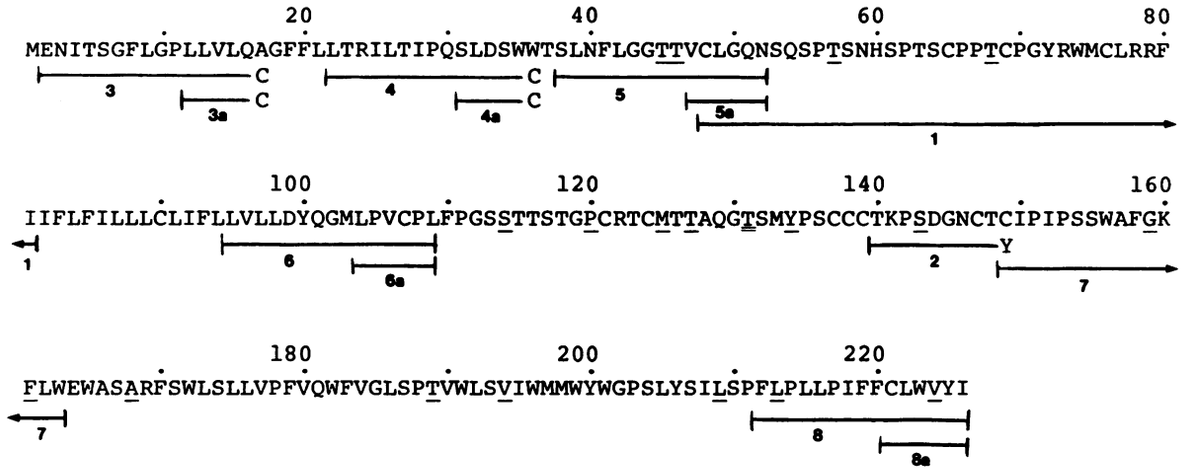

FIG. 1. The 226 amino acid sequence of HB_SAg as translated by Pasek *et al.* (9) from the nucleic acid sequence. Regions of the protein chosen for synthesis are indicated by bold underlining and numbered 1–8 or 3a–6a, 8a. C or Y at the end of a bold underline indicates the addition of cysteine or tyrosine not found in the primary sequence. Residues that are not the same in all three nucleotide sequence determinations (8–10) are lightly underlined. Many of these cluster at 110–140. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

subjected to acid hydrolysis at reduced pressure (6 M HCl, 110°C, 72 hr), and its amino acid composition was determined. No attempt was made to remove multimeric forms because the sole use of the peptides was as immunogens.

Coupling of Synthetic Peptides to Carrier Protein. All peptides except 1, 3a, and 7 were coupled to the carrier protein keyhole limpet hemocyanin (KLH) through the cysteine of the peptide by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as the coupling reagent (13, 14). In general, 5 mg of peptide in phosphate-buffered saline (P_i/NaCl) (pH 7.5) or sodium borate buffer (pH 9.0) was coupled to 3–4 mg of KLH–MB. The pH for dissolving the peptide was chosen to optimize its solubility and content of free cysteine [determined by Ellman's method (15)]. For each peptide, 5 mg of KLH in 0.25 ml of 0.05 M P_i, pH 6, was treated with MBS dimethylformamide at KLH/MBS = 1:40, stirring for 30 min at room temperature. The KLH–MB was then passed through Sephadex G-25 and washed with 0.05 M P_i, pH 6, to remove free MBS; KLH recovery from the peak fractions of the column eluate (monitored by A_{280}) was estimated as ≈80%. The KLH–MB was then treated with 5 mg of peptide at pH 7–7.5, stirring for 3 hr at room temperature. Coupling efficiency was monitored with radioactive peptide. In general, 25–50 molecules of peptide were coupled per 100,000 daltons of KLH.

Preparation of Antipeptide Antibodies. Rabbits were immunized with peptide-coupled KLH according to the following schedule: (*i*) 200 μg in complete Freund's adjuvant (1:1) subcutaneously on day 0, (*ii*) 200 μg in incomplete Freund's adjuvant (1:1) subcutaneously on day 14, (*iii*) 200 μg with 4 mg of alum intraperitoneally on day 21 and day 91. Animals were bled 4 and 15 weeks after the first injection. Peptide 1 was injected without KLH (1 mg per injection) according to the same schedule.

Immune Precipitation of Synthetic Peptides. The reactivity of the various antipeptide sera was determined by their ability to immunologically precipitate radioiodinated target proteins. Peptides were labeled with ^{125}I by the chloramine T reaction if they contained tyrosine or with Bolton–Hunter reagent. Highly purified envelope preparations (a gift from J. Gerin) were labeled by using chloramine T. Radioiodinated targets were either suspended in P_i/NaCl or in RIPA buffer (0.15 M NaCl/10 mM sodium phosphate, pH 7.5/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% $NaDodSO_4$) and treated (5×10^6 cpm per reaction) at 0°C with 5 μl of test serum or normal rabbit serum for 1 hr; precipitates were collected with *Staphylococcus aureus*. Pellets were washed once with RIPA buffer and then twice with 500 mM LiCl/100 mM Tris (pH 8.5) and assayed for radioactivity. Variability was ≈20% in duplicate determinations.

Polyacrylamide Gel Electrophoresis of Immune Precipitates. Purified Dane particles (a gift of W. Robinson) were suspended in RIPA buffer and radioiodinated with chloramine T. The preparation was precleared twice by incubation at 0°C with normal rabbit serum for 30 min and then with formalin-fixed *S. aureus* for 30 min, followed by centrifugation (5 min at 12,000 × *g*); it was then incubated with 5 μl of normal antipeptide 3 or antipeptide 4 serum. Precipitates were collected and washed as above, suspended in gel loading buffer, boiled, centrifuged to remove *S. aureus*, and subjected to electrophoresis on a 5–17% acrylamide/$NaDodSO_4$ gel and autoradiographed.

RESULTS

Selection of Peptides for Synthesis. Considerations concerning the physical structure of the HB_SAg, as well as variations among the three published nucleotide sequences, dictated the selection of peptides for chemical synthesis. In general, we tried to select regions so as to span as large a portion of the protein sequence as possible and also include a cysteine residue to allow coupling to a carrier protein. If the nucleotide sequence did not predict a cysteine in a region of interest, one was added to the COOH terminus. The peptides synthesized are underlined in Fig. 1 and listed in Table 1.

We did not cover the entire protein because we judged some regions less likely to succeed than others. We avoided the area between 81 and 94 because of its extreme hydrophobicity. We did not expect synthetic peptides corresponding to this sequence to be soluble and, even if an antibody to them could be raised, one would not expect this region to be located on the surface of the native protein. For similar reasons, we did not study a large portion (positions 164–211) of the hydrophobic COOH terminal domain. We avoided the region between positions 110 and 140 because there was not a consensus in this region among the published nucleotide sequences (8–10). Pep-

Table 1. Peptide sequence and position (corresponding to the underlined residues in Fig. 1)

Fragment	Position	Sequence
1	48–81	Cys-Leu-Gly-Gln-Asn-Ser-Gln-Ser-Pro-Thr-Ser-Asn-His-Ser-Pro-Thr-Ser-Cys-Pro-Pro-Thr-Cys-Pro-Gly-Tyr-Arg-Trp-Met-Cys-Leu-Arg-Arg-Phe-Ile
2	140–148	Thr-Lys-Pro-Ser-Asp-Gly-Asn-Cys-Thr-*Tyr*
3	2–16	Glu-Asn-Ile-Thr-Ser-Gly-Phe-Leu-Gly-Pro-Leu-Leu-Val-Leu-Gln-*Cys*
3a	12–16	Leu-Leu-Val-Leu-Gln-*Cys*
4	22–35	Leu-Thr-Arg-Ile-Leu-Thr-Ile-Pro-Gln-Ser-Leu-Asp-Ser-Trp-*Cys*
4a	31–35	Ser-Leu-Asp-Ser-Trp-*Cys*
5	38–52	Ser-Leu-Asn-Phe-Leu-Gly-Gly-Thr-Thr-Val-Cys-Leu-Gly-Gln-Asn
5a	47–52	Val-Cys-Leu-Gly-Gln-Asn
6	95–109	Leu-Val-Leu-Leu-Asp-Tyr-Gln-Gly-Met-Leu-Pro-Val-Cys-Pro-Leu
6a	104–109	Leu-Pro-Val-Cys-Pro-Leu
7	149–163	Cys-Ile-Pro-Ile-Pro-Ser-Ser-Trp-Ala-Phe-Gly-Lys-Phe-Leu-Trp
8	212–226	Phe-Leu-Pro-Leu-Leu-Pro-Ile-Phe-Phe-Cys-Leu-Trp-Val-Tyr-Ile
8a	221–226	Cys-Leu-Trp-Val-Tyr-Ile

Residues shown in italic were not in the primary protein sequence but were added to allow coupling to carrier or radioiodination. All peptides except 1, 3a, and 7 were coupled to carrier protein KLH as described in *Materials and Methods*. Peptide 1 was used without coupling to KLH. Peptides 3a and 7 were insoluble and not used.

tides corresponding to the extreme NH_2 and COOH termini of the molecule were included because of previous success in using these regions of proteins where the complete tertiary structure was unknown (2, 3). The remaining peptides were selected to correspond to hydrophilic domains of the protein, as well as to proline-containing junctions between hydrophilic and hydrophobic domains where the protein might be expected to turn and expose "corners." Peptides 3a and 7 were found to be insoluble and, hence, were not pursued.

Antibodies to Some Synthetic Peptides React with Native HB_SAg. Before testing reactivity to native HB_SAg, it was important to ensure that an antibody response to the synthetic peptide had occurred. As seen from the data in Table 2, when coupled to KLH, 6 of the 10 peptides were immunogenic as judged by the ability of the antisera to precipitate the radioiodinated peptides. Only peptides 4a, 8, and 8a failed to elicit an antipeptide response. Peptide 2 elicited only a marginal response. Peptide 1 was an effective immunogen without coupling to KLH. Although the extent was graded with time, early bleeds indicated the direction of the responses.

To determine whether the antibodies raised against the various peptides could react with HB_SAg, we assayed their ability to immunoprecipitate radioiodinated HB_SAg that had been purified from hepatitis B Dane particles. When the HB_SAg was suspended in RIPA buffer, four of the seven antibodies that reacted against the appropriate peptide also precipitated HB_SAg. Specifically, antibodies to peptides 1, 3, 4, and 6 reacted with purified HB_SAg, whereas antibodies to peptides 5, 5a, and 6a failed to react, as did those antisera that did not see their target peptide (4a, 8, and 8a). Again, peptide 2 antisera gave a marginal reactivity. There is variability among sera of rabbits that had received identical treatments. In all but one animal, no. 03302, the early results were predictive of the 3-month response. Peptide 8 was not very soluble and thus the failure of two rabbits to respond to it must be considered tentative in light of our inability, because of solubility, to determine how efficiently it coupled to KLH.

Several interesting features concerning individual peptides as immunogens are evident in Table 2. Peptide 6 is highly immunogenic and induces antibody reactive with itself as well as with native HB_SAg. However, peptide 6a (the COOH-terminal six amino acids of peptide 6), although capable of inducing antibody to itself, does not induce antibody reactive with native HB_SAg. On the other hand, peptide 4 is capable of inducing antibody to itself and native HB_SAg, whereas the COOH-terminal hexamer (peptide 4a) does neither. Peptide 1 is of special interest from two points of view. First, its immunogenicity does not depend on a carrier, perhaps because it is of sufficient length to induce antibody by itself. But, more interesting is the fact that its ability to induce antibody reactive with native HB_SAg depends on the pH used to solubilize the immunogen. At pH 5.3, peptide 1 is completely soluble and expresses 62% free cysteine. Antibodies raised against the peptide solubilized at this pH recognize the target peptide as well as the native

Table 2. Reactivity of antipeptide sera against peptide and viral envelope

		Antibody titer			
		Versus peptide		Versus viral envelope	
Peptide	Rabbit	4 weeks	15 weeks	4 weeks	15 weeks
1*	03288	6.4	8.4	8.3	13.4
1*	03289	8.6	7.6	28.0	52
1†	03300	3.7	—	1.0	—
2	03370	2.1	1.3	2.4	1.0
2	03371	2.7	1.7	1.1	0.9
3	03302	1.6	20	2.0	5.8
3	03303	5.2	15.8	14.0	36
3a‡					
4	03220	7.9	7.5	32.5	92
4	03221	4.8	6.1	7.2	71
4a	03211	1.0	—	1.0	—
4a	03213	1.0	—	1.0	—
5	03308	8.5	—	1.0	—
5	03310	5.9	—	1.0	—
5a	03305	5.3	—	1.0	—
5a	03307	5.8	—	1.0	—
6	03306	51.0	85	75.6	113
6	03309	17.7	83	9.5	37
6a	03169	12.3	—	1.0	—
6a	03212	11.0	25	1.0	1.0
7‡					
8	03219	1.0	—	1.0	—
8	03210	1.0	—	1.0	—
8a	03215	1.0	—	1.0	—
8a	03216	1.0	—	1.0	—

Antibody titer is expressed as radioactivity (cpm) precipitated by test serum divided by radioactivity precipitated by normal serum.
* Injected at pH 5.3.
† Injected at pH 8.5.
‡ Insoluble.

HB_SAg. In contrast, at pH 8.5, the peptide is barely soluble (less than 15%) and expresses no free cysteine. When injected at this pH, the peptide elicits a poor response to itself and none to HB_SAg.

Although RIPA buffer would not be expected to denature HB_SAg, we wished to study the immune reactivity of the protein under conditions more like physiological. Accordingly, the antigen was suspended in P_i/NaCl and treated with various antipeptide sera. All sera reacted with the HB_SAg in P_i/NaCl with the same efficiency as in RIPA buffer (data not shown). Thus, the antibodies recognize the protein under conditions that approximate its native condition. Therefore, antibodies against such peptides might be expected to function *in vivo* as well as *in vitro*.

To determine which protein(s) of Dane particles were reactive with antibodies to these synthetic peptides, purified Dane particle (serotype adw) were disrupted with detergent and the proteins were radioiodinated. The labeled proteins were precipitated with the various antipeptide sera, and the components present in the precipitates were analyzed on $NaDodSO_4$/polyacrylamide gels. Two major components with approximate sizes of 28,000 and 23,000 daltons were specifically precipitated from Dane particles by antibodies reactive with native HB_SAg (Fig. 2). The 28,000 and 23,000 dalton species correspond to the previously described (6, 7) major forms of HB_SAg (I and II), which differ in their degree of glycosylation. In addition, antisera against peptide 3 (Fig. 2) and peptide 6 (data not shown) also reacted with proteins of ≈47,000 and 170,000 daltons, which presumably represent multimeric forms or precursor molecules. The 47,000-dalton species is most likely the dimeric form of HB_SAg (16, 17). Thus, the antibodies are directed against a protein found in the known etiologic agent of hepatitis B.

The concept of proteins that are precursors to HB_SAg is consistent with the data of Robinson in which tryptic digest patterns show that some spots of the higher M_r forms correspond to those of HB_SAg whereas other spots do not (W. Robinson, personal communication). Whereas all antibodies reactive with HB_SAg see the 23,000- and 28,000-dalton forms of HB_SAg in Dane particles, only some see the higher M_r forms. Presumably, the conformation or the degree of glycosylation (or both) of the larger forms is such that the peptide in question is hidden. Alternatively, the processing of the precursor may include binding to proteins or cellular structures that hide the target peptide.

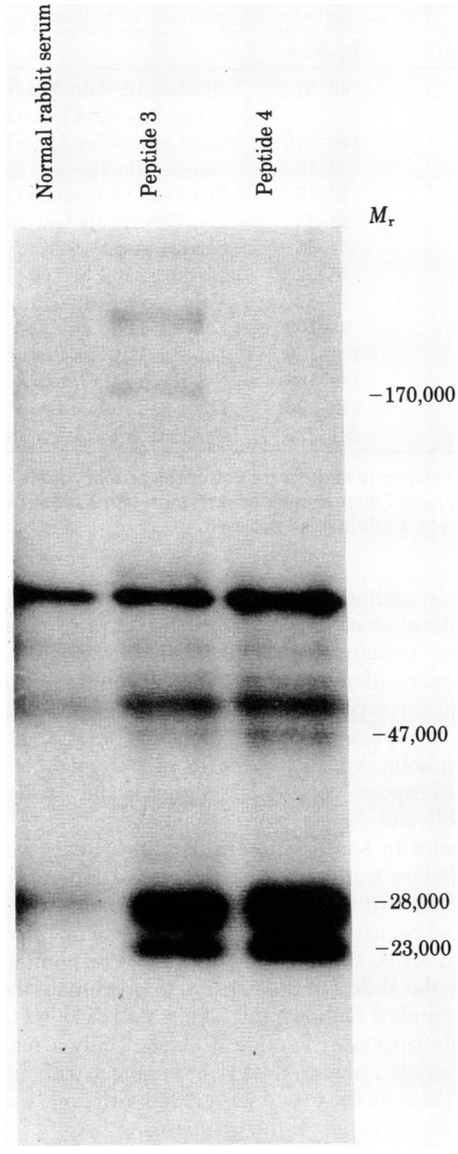

FIG. 2. Radioactively labeled, purified Dane particles were treated with 5 μl of normal antipeptide 3 or 4 serum. Precipitates were collected and prepared for electrophoresis as described in *Materials and Methods*. Samples were subjected to electrophoresis on a 5–17% $NaDodSO_4$/polyacrylamide gel and autoradiographed.

DISCUSSION

In broad outline, this paper illustrates that one can take a given nucleotide sequence, chemically synthesize several peptides from various domains of the predicted protein, and, with some of these, raise antibodies reactive with the native structure. As the hepatitis protein had not been chosen for study because of any structural feature suggesting unique oportunities for success, our results suggest that the strategy is general and should work for any protein as long as enough domains are studied. As for the "rules" we have learned to date, peptides of limited solubility or containing fewer than six amino acids are a poor choice. We noticed that all the productive peptides contained one or more prolines, a fact consistent with its known presence in turns. In our study, four of six soluble peptides, ranging from 10 to 34 residues, proved useful. We consider this encouraging in terms of the general application of this technique to finding unknown proteins from the known nucleotide sequence of their gene.

Previous studies of HB_SAg concluded that it is critically dependent on conformation for preparation of antibodies reactive with the native structure. Vyas and colleagues suggested that reduction and alkylation of the disulfide bonds of the hepatitis B antigen resulted in complete loss of antigenicity (18). By contrast, our results show that there are determinants in the HB_SAg that are not dependent on any conformation other than that which can be attained by short peptides. When the two studies are considered together, one concludes that a linear sequence as part of a larger denatured structure, albeit alkylated, will not elicit antibodies reactive with the native molecule whereas that same sequence free from constraints of neighboring amino acids will elicit such antibodies. Sachs *et al.* (19) have provided a theoretical framework in which such results can be considered. Basically, the argument is that a peptide in solution exists in an equilibrium between all possible conformations, some of which correspond to those present in the native molecule. Depending on the equilibrium constraints, more or less of the peptide would be expected to be in a "native" conformation at any given time, but one would expect that, when an animal is injected with a peptide, all possible conformations will be presented. Con-

versely, polypeptides constrained by adjacent sequences in denatured proteins do not enjoy a degree of freedom consistent with presentation of all conformational states to the animal. This leads one to conclude that the success in eliciting antibodies reactive with native molecules depends not only on factors such as solubility and surface localization of the peptide but also on the percentage of time a peptide in solution exists in its native conformation. Thus, the utility of proline may be a reflection of the fact that one bond within the peptide has a frozen angle.

There are domains of the HB_SAg that remain to be explored by using synthetic polypeptides. We know little about the protein at positions 110–140 and 162–210. The hydrophilic region between 110 and 140 is of particular interest because of the high degree of variation among the various different sequences. Interestingly, in the study by Pasek *et al.* (9), the plasma used as a source of Dane particles was of complex serotype (adw and agw), and these authors noted microheterogenicity in the region of sequence corresponding to HB_SAg. Perhaps the sequence variation at 110–135 corresponds to the domain of the molecule conferring type specificity to the HB_SAg. A definitive way of settling the issue of the structural basis for different serotypes would be to take antibodies to a given sequence in this region and test them against HB_SAg of different serotypes. The 40–50 region also shows significant heterogeneity among the three nucleotide sequences. In this regard, it is of interest that antibodies made against peptide 5, which corresponds to the region predicted from the nucleotide sequence of Pasek *et al.*, do not react with our test envelope (serotype adw). This may be due to the fact that, in this region, perhaps a second region of type-specific variation, the peptides we chose did not correspond to that of the envelope we used.

The results presented here establish the generality that one can synthesize peptides predicted from nucleic acid sequences and raise antibodies reactive with the native molecule. Such antibodies are unique reagents insofar as they react with a small region of the native molecule that is known in advance. Thus, antibodies made in this way differ from hybridomas which, although useful at the outset for studies of whole proteins, must be further characterized for fine-structure analysis of domains.

The preparation of antibodies against protein fragments or synthetic peptides that will neutralize virus (for review, see ref. 20) or even bacterial toxins (21) is well documented. We expect that, in the future, most new information concerning the structure of biologically important proteins will be generated by nucleic acid sequence analysis. Synthetic peptides prepared by using nucleotide sequences as patterns should be ideal for use in vaccination. For example, a combination of polypeptides (such as 1, 3, 4, and 6) might provide broad protection against hepatitis B virus, thereby obviating biological variables such as serotypic diversity, antigenic drift of the infectious agent, and the individuality of the host immune response.

We are grateful to Drs. James Bittle, Paul Janssen, John Elder, Ron Ogata, Henry Niman, and Frank Dixon for helpful discussions. We thank Dr. John Gerin for providing purified hepatitis B surface antigen and Dr. William Robinson for providing purified Dane particles. We thank Drs. Jack Kyte and Russell Doolittle for making their excellent computer programs available to us prior to publication. This research was supported by a grant from the Pitman-Moore Company of Johnson and Johnson. This is publication no. 2378 from the Research Institute of Scripps Clinic.

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