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2 June 1982; revised 9 August 1982

Herpes Simplex Virus Type-1 Glycoprotein D Gene: Nucleotide Sequence and Expression in *Escherichia coli*

Abstract. *The protein coding region of the herpes simplex virus type-1 glycoprotein D (gD) gene was mapped, and the nucleotide sequence was determined. The predicted amino acid sequence of the gD polypeptide was found to contain a number of features in common with other virus glycoproteins. Insertion of this protein coding region into a bacterial expressor plasmid enabled synthesis in* Escherichia coli *of an immunoreactive gD-related polypeptide. The potential of this system for preparation of a type-common herpes simplex virus vaccine is discussed.*

The closely related herpes simplex type-1 and type-2 viruses (HSV-1 and HSV-2) are the causative agents of various primary and recurrent human diseases ranging in severity from minor skin infections through often fatal complications (in exposed newborns) and viral encephalitis (*1*). In addition, there may be as many as 9 million cases of recurrent genital infections caused primarily by type-2 each year in the United States alone (*2*).

The infectious HSV particles consist of a linear double-stranded DNA genome of 150 kilobase pairs (kbp) in length contained within an icosahedral nucleocapsid that is in turn surrounded by a membrane envelope (*3*). Embedded within the virion envelope, which is derived from the cellular lipid bilayer during maturation of the virus, are five major HSV-specified glycoproteins, designated gA, gB, gC, gD, and gE (*4*). These virus glycoproteins, with the exception of gA and gB, are antigenically distinct and are required for penetration of the virus into the cell, virus-induced cell fusion, and probably other aspects of virus replication (*5*). Antiserums to each of these glycoproteins can neutralize infectivity of the homologous HSV type in an in vitro assay (*4*, *6*). Whereas antiserums to gC and gE are specific for the HSV type to which they were directed (*6*, *7*), antiserum prepared to HSV-1 gD polypeptide is type-common. Thus, polyvalent gD antiserum (*8*) and certain gD monoclonal antibodies (*9*, *10*) recognize antigenic determinants (epitopes) and neutralize infectivity of both HSV-1 and HSV-2.

The type-common specificity of gD antiserum was demonstrated in vivo by passively immunizing mice with a monoclonal antibody directed against HSV-1 gD (*11*). Such mice were protected against acute neurological disease induced by either HSV-1 or HSV-2. This observation further demonstrated the potential for inducing immunity to infections of both HSV types with the use of a subunit vaccine consisting of a purified HSV-1 gD polypeptide. To this end, we have characterized the genome location and sequence of the HSV-1 DNA encoding gD. Here, we describe the nucleotide sequence of an HSV-1 DNA fragment containing the entire gD polypeptide coding region. Fusion of this coding region with the *lac* promoter enabled synthesis of an immunoreactive gD-related polypeptide in *Escherichia coli*.

The HSV gD gene was mapped by intertypic recombinant analyses (*12*) between 0.9 to 0.945 genome map units (Fig. 1A) in the single-copy short (U_s) region of the virus DNA [for a detailed review of the HSV DNA structure, see (*13*)]. We have mapped the gD gene more precisely by two techniques: (i) by selecting messenger RNA (mRNA) species by hybridization to specific HSV-1 DNA fragments and translation of these mRNA's in vitro and (ii) by expressing cloned HSV-1 DNA fragment by injection into the nuclei of frog oocytes (*14*). We concluded that gD was encoded by a 3.0-kilobase (kb) mRNA, mapping predominantly in a 2.9-kbp Sac I DNA fragment subsequently cloned in plasmid pSC30-4 (Fig. 1B). A recent report by

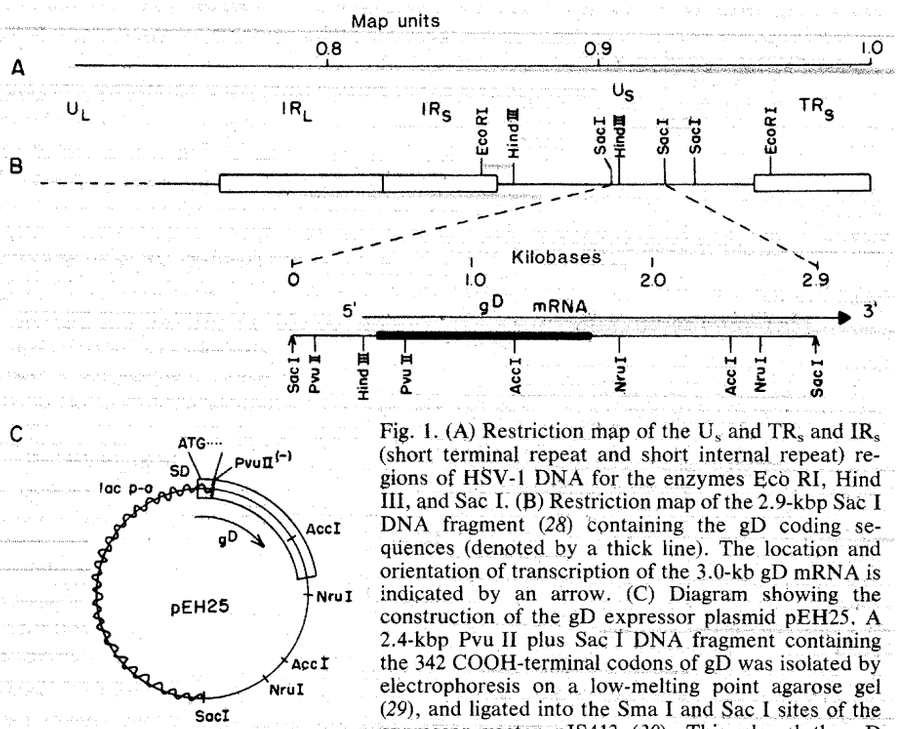

Fig. 1. (A) Restriction map of the U_s and TR_s and IR_s (short terminal repeat and short internal repeat) regions of HSV-1 DNA for the enzymes Eco RI, Hind III, and Sac I. (B) Restriction map of the 2.9-kbp Sac I DNA fragment (*28*) containing the gD coding sequences (denoted by a thick line). The location and orientation of transcription of the 3.0-kb gD mRNA is indicated by an arrow. (C) Diagram showing the construction of the gD expressor plasmid pEH25. A 2.4-kbp Pvu II plus Sac I DNA fragment containing the 342 COOH-terminal codons of gD was isolated by electrophoresis on a low-melting point agarose gel (*29*), and ligated into the Sma I and Sac I sites of the expressor vector pJS413 (*30*). This placed the gD coding region in the correct reading frame downstream from a modified λ *cro* sequence (*26*) containing the Shine and Dalgarno sequence (*SD*), the initiation sequence (*ATG*), and a further 23 codons. HSV-1 DNA sequences are represented by a smooth circular line and those derived from pJS413 by a wavy line. These coding sequences (indicated by an open circular box) are transcribed in the direction indicated by the arrow under the control of the *E. coli lac* promoter (*lac p-o*). This plasmid, pEH25, was used to transform *E. coli* strain NF1829 (*31*).

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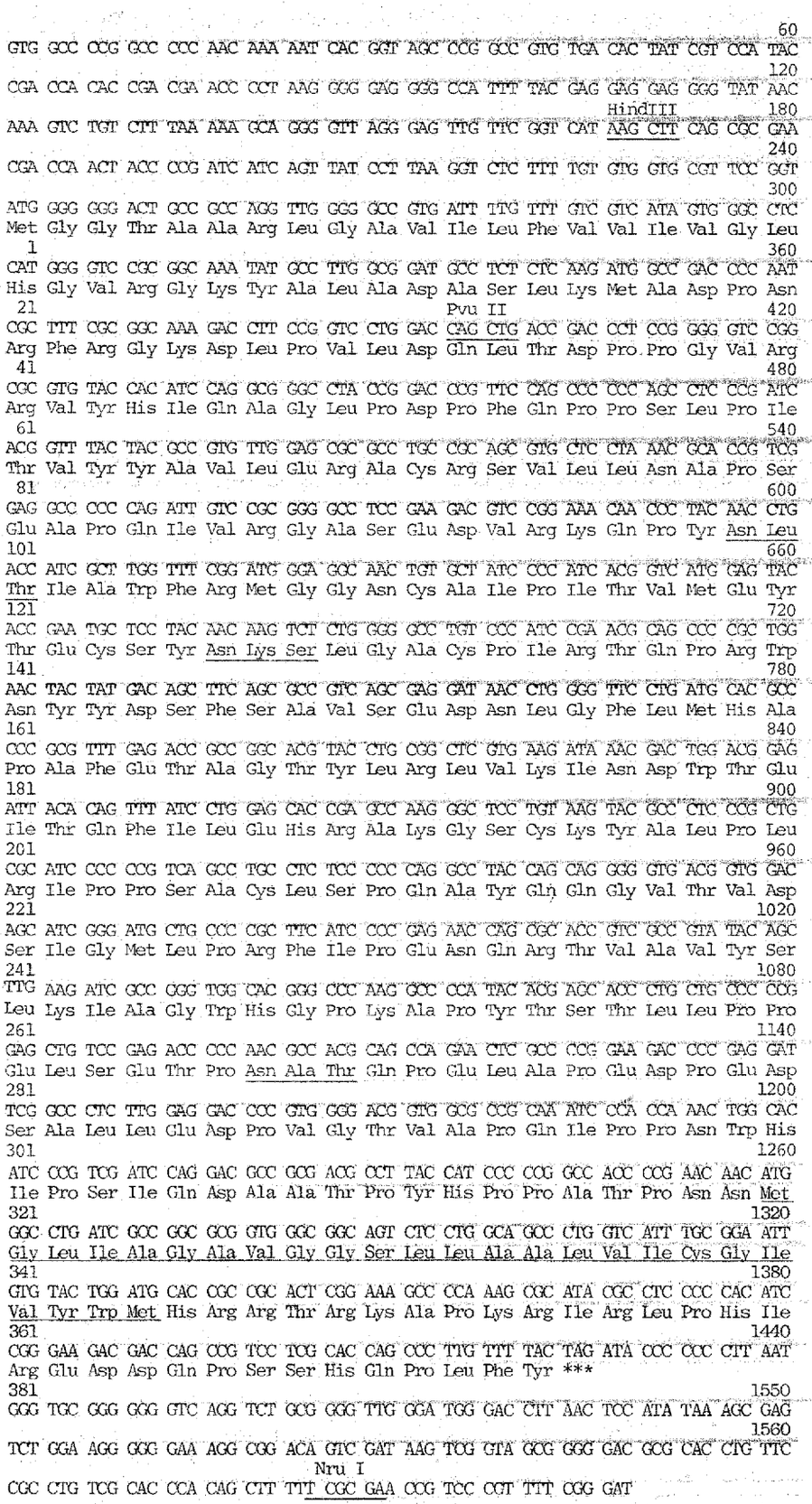

Fig. 2. Nucleotide sequence of the coding region of the HSV-1 gD gene. The DNA sequence of plasmid pSC30-4 was determined for both strands (*32*). Represented here is the sequence of the noncoding strand only. Nucleotides are numbered to the right of the DNA sequence, which is transcribed from left to right. The positions of the Hind III, Pvu II, and Nru I cleavage sites are indicated. The predicted gD polypeptide amino acid sequence is numbered at the left. Underscored are the potential sites for glycosylation (amino acids 118 to 121, 146 to 148, and 287 to 289) and the proposed α-helical membrane-spanning region (amino acids 340 to 364).

Lee *et al.* (*15*) also demonstrated that the gD-1 gene maps within this Sac I DNA fragment. The S1 nuclease analyses (*14*) indicated that the 5′ terminus of the gD mRNA mapped very close to the Hind III site within this Sac I DNA fragment (Fig. 1B) and, further, that the gD gene was uninterrupted by noncoding intervening sequences. The absence of intervening sequences in the HSV-1 gD gene made it possible to directly insert a suitable HSV-1 DNA sequence into a bacterial expressor vector (pJS413) to gain synthesis of a gD-related polypeptide.

To facilitate insertion of gD coding sequences into pJS413 in the correct reading frame, the nucleotide sequence of the entire gD coding region and DNA 5′ to the mRNA terminus was determined (Fig. 2) (*16*). Nuclease mapping data indicated that the gD mRNA 5′ terminus was located between residues 156 to 164 (Fig. 2). The sequences TTTAAAA (residues 131 to 138) (*17*) and GGCCATTT (residues 92 to 99) preceding the presumed mRNA 5′ terminus may correspond to the transcriptional regulatory TATA and CAT signals, respectively (*18, 19*). The first ATG in the transcribed DNA sequence is located at residues 241 to 243 (Fig. 2); this reading frame is open for a further 393 codons. As the ATG nearest the 5′ end in a transcribed DNA sequence corresponds most commonly to the protein synthesis initiator (*20*), we expect that gD polypeptide synthesis initiates at this codon. Moreover, subsequent ATG sequences located in the alternative reading frames are soon followed by in-frame termination codons.

The amino acid sequence of the gD polypeptide predicted from the nucleotide sequence is presented in Fig. 2. The molecular weight of the unmodified polypeptide was found to be 43291 and was notably rich in proline residues (43 out of a total of 394 amino acids). The presence of many α-helical–disrupting residues may explain why the molecular weight of the presumptive gD precursor (50,000) estimated by gel electrophoresis (*21*) is rather greater than that found here. The first 20 NH_2-terminal amino acids, with the exception of arginine at position 7, are hydrophobic or nonpolar. This sequence probably corresponds to a signal peptide for membrane insertion and, as such, may be removed during translation (*22*). A further strongly hydrophobic region of 25 amino acids is present at positions 340 to 364 (Fig. 2) and has the characteristics of a membrane-spanning α-helical region (*23*). This putative transmembrane sequence

is followed by a strongly basic region, which probably serves to anchor the glycoprotein in the membrane (*23*). If these assumptions are correct, the major NH_2-terminal portion of the gD would be positioned external to the membrane. Three potential glycosylation sites (Asn-X-Ser or Asn-X-Thr) (*24*) are present in the presumptive external part of the protein as indicated in Fig. 2.

The presence of a signal peptide sequence was conditionally lethal for expression of the vesicular stomatitis virus glycoprotein gene in *E. coli* (*25*). In our construction of a gD-expressor plasmid, therefore, we took advantage of the Pvu II site (residue 396) located 52 codons from the ATG at residues 241 to 243 (Fig. 2). Ligation of the 2.4-kbp Pvu II and Sac I DNA fragment, as outlined in Fig. 1C, into the Sma I and Sac I sites of pJS413 resulted in deletion of sequences encoding the NH_2-terminal 52 amino acids of gD. This construction brought the remaining 342 COOH-terminal gD codons in-frame with a 24 codon NH_2-terminal bacteriophage λ *cro* sequence, containing an initiating ATG, placed under the transcriptional control of the *lac* promoter (*26*). This construction was used to transform *E. coli* strain NF1829. A plasmid, pEH25, was isolated which, by restriction endonuclease cleavage analysis and sequencing of the Sma I–Pvu II junction, had the expected structure. The *E. coli* strain NF1829 is an overproducer of the *lac* repressor. Hence, in this strain the *lac* promoter of pEH25 was transcriptionally silent unless induced by lactose or galactoside analogs, such as isopropyl-β-D-thiogalactopyranoside (IPTG).

To test for expression of a gD-related protein, pEH25 transformants were incubated with [^{35}S]methionine under conditions in which transcription from the *lac* promoter was either induced or uninduced. Labeled polypeptides were extracted and subjected to immunoprecipitation after the addition of a number of different monoclonal antibodies directed against HSV-1 gD (*9*), and with other control serums. Immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Of these monoclonal antibodies, 1S, 55S, and 57S were found to immunoprecipitate a polypeptide of apparent molecular weight 46,000 specifically from induced cell lysates (Fig. 3B, lanes 4, 6, and 8). This protein was immunoprecipitated also by polyvalent antiserum directed against whole HSV-1 (Fig. 3A, lane 6). Control serums (serum from a rabbit that was not immunized and a monoclonal antibody specific for fibronectin) did not immunoprecipitate the 46,000 molecular weight protein (Fig. 3A, lane 4; Fig. 3B, lane 2). Plasmid isolates in which the gD gene was fused with the pJS413 *lac* plus *cro* sequence in the incorrect reading frame were negative for the induction of the 46,000 molecular weight protein (data not shown). The monoclonal antibodies, but not the polyvalent antiserum to HSV-1, precipitated smaller polypeptides of molecular weight 36,000 to 37,000 in addition to the 46,000 molecular weight protein. These smaller polypeptides may indicate premature termination or reinitiation events. The 46,000 molecular weight protein was not subject to rapid proteolysis in *E. coli*, as the immunoprecipitations were performed with proteins labeled for 1 hour—that is, representing a steady state of synthesis.

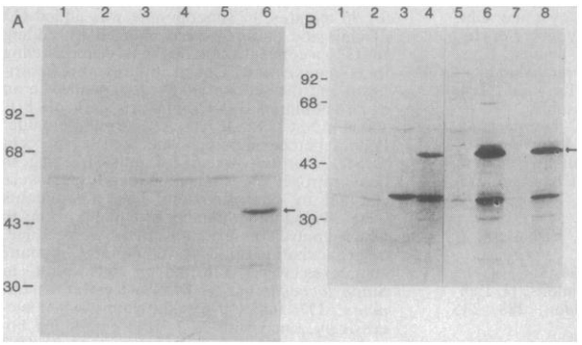

Fig. 3. Immunoprecipitation of the 46,000 molecular weight gD-related polypeptide from pEH25 transformant extracts (*33*). (A) Uninduced (lanes 1, 3, and 5) or IPTG-induced cultures (lanes 2, 4, and 6) were immunoprecipitated with no added antibody (lanes 1 and 2), serum from an unimmunized rabbit (lanes 3 and 4), and rabbit antiserum to HSV-1 (lanes 5 and 6). (B) Uninduced (lanes 1, 3, 5, and 7) or IPTG-induced cultures (lanes 2, 4, 6, and 8) were immunoprecipitated with a control fibronectin monoclonal (lanes 1 and 2) and with gD monoclonal antibodies 1S (lanes 3 and 4), 55S (lanes 5 and 6), and 57S (lanes 7 and 8). To the left of the fluorographs the positions of molecular weight standards (30, 43, 68, and 92 K) which were subjected to coelectrophoresis with the immunoprecipitates are indicated. Arrows indicate the location of the 46,000 molecular weight gD-related polypeptide.

The above experiments indicated that pEH25, upon induction, specified a gD-related protein containing the epitopes for the 1S, 55S, and 57S monoclonal antibodies. The antibodies are all specific for HSV-1, and 1S neutralizes virus infectivity in vitro, whereas 55S and 57S do not (*9*). Recent experiments with other HSV-1 gD-directed monoclonal antibodies (*9*) indicated that the 46,000 molecular weight protein induced in pEH25 transformants was precipitated also by 4S monoclonal antibody (an HSV type-common neutralizing antibody), but not by other monoclonal antibodies (11S, 12S, and 50S) differing in their HSV type-specificity and neutralizing activity (data not shown).

By Coomassie blue staining of SDS-PAGE gels, it was estimated that the gD-related polypeptide induced in pEH25 transformants represented approximately 0.1 percent of the total *E. coli* cellular protein. Further plasmid constructions, in which the gD coding region was ligated (in-frame) to the entire β-galactosidase coding sequence, have resulted in induction of a fusion protein comprising more than 5 percent of the total cell protein (*27*). We now have evidence that rabbits injected with this latter fusion protein elicit neutralizing antibody to HSV-1 and HSV-2. This system represents a means by which a safe, inexpensive, and potentially effective vaccine against both HSV-1 and HSV-2 may be produced.

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U, uracil; abbreviations of the amino acid residues are: Ala, alanine; Asn, asparagine; Asp, aspartic acid; Arg, arginine; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

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33. To label proteins, cultures were grown to stationary phase overnight at 37°C in L broth, then diluted 20-fold in M-9 broth [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1972).] After further incubation at 37°C for 90 minutes, [^{35}S]methionine was added (25 μCi/ml) and cultures were induced by adding IPTG to a concentration of 1 m*M*. After labeling for 60 minutes at 37°C, cell cultures were centrifuged and the sediment was resuspended in an equal volume of IP-3 (20 m*M* tris-HCl, *p*H 8.1, 100 m*M* NaCl, 1 m*M* EDTA, 1 percent Nonidet P-40, 1 percent deoxycholate, and 0.1 percent SDS). After two cycles of quick-freezing in liquid nitrogen and sonication, cell lysates were clarified by centrifugation. The supernatants were divided into a number of equal portions to which control or test antiserums were added. After incubation on ice for 60 minutes, immune complexes were collected by adsorption to *Staphylococcus aureus* [S. W. Kessler, *J. Immunol.* **177**, 1482 (1976)] and were washed successively, once with IP-2 (IP-3 containing bovine serum albumin, 20 mg/ml), once with IP-2 containing 1*M* NaCl, twice with IP-3, and once with IP-1 (20 m*M* tris-HCl, *p*H 8.1, 100 m*M* NaCl, 1 m*M* EDTA, 1 percent NP-40). Finally, pellets were resuspended in SDS-PAGE sample buffer [U. K. Laemmli, *Nature (London)* **227**, 680 (1970)], heated at 95°C for 2 minutes, then were clarified by centrifugation and loaded onto a 10 percent SDS-polyacrylamide gel. After electrophoresis, proteins were visualized by staining with Coomassie blue dye, then treated with 1*M* sodium salicylate and dried for fluorography. Films were, in general, exposed overnight at −70°C.
34. We thank Berge Hampar and Martin Zweig of the National Cancer Institute, Frederick, Md., for providing HSV-1 monoclonal antibodies used in this study, Tom Silhavy for the *E. coli* strain NF1829, and Bob Stallard for assistance with DNA sequencing. The collaboration of Anamaris Colberg-Poley, Carol Marcus-Sekura and Barrie Carter of the National Institutes of Health, Bethesda, Md., in the oocyte injection experiments is gratefully acknowledged.

7 July 1982; revised 30 August 1982

Heritable True Fitness and Bright Birds: A Role for Parasites?

Abstract. *Combination of seven surveys of blood parasites in North American passerines reveals weak, highly significant association over species between incidence of chronic blood infections (five genera of protozoa and one nematode) and striking display (three characters: male "brightness," female "brightness," and male song). This result conforms to a model of sexual selection in which (i) coadaptational cycles of host and parasites generate consistently positive offspring-on-parent regression of fitness, and (ii) animals choose mates for genetic disease resistance by scrutiny of characters whose full expression is dependent on health and vigor.*

Whether mate choice could be based mainly on genetic quality of the potential mate has been a puzzle to evolutionary biologists (*1, 2*). Population genetic theory predicts that any balanced polymorphism for a selected trait ends with zero heritability of fitness, so that no one mate is better for "good genes" than any other. However, females of many species act as if they are choosing males for their genes; thus "good genes" versions of sexual selection have been frequently, albeit tentatively, suggested (*2–4*). Here we propose a way out of the difficulty via a previously unconsidered mechanism of sexual selection, and give preliminary evidence for the operation of the principle in North American birds.

There may exist a large class of genes with effects on fitness that always remain heritable. The genes are those for resistance to various pathogens and parasites. The interaction between host and parasite [parasite here being interpreted in a broad evolutionary sense (*5*)] is unusual because it so very readily produces cycles of coadaptation. These cycles can ensure a continual source of fitness variation in genotypes.

To illustrate, imagine a host and parasite population, each with the two alternative genotypes *H*, *h* and *P*, *p*, respectively. For simplicity, assume the organisms are haploid, although diploid models can easily and realistically be made to work in a similar fashion. An *H* individual is resistant to pathogen type *p*, but susceptible to *P*, and vice versa for *h* individuals. The parasite, of course, flourishes in an individual host that is susceptible and dies (or is less productive) in a host that is resistant.

If a female chooses an *H* male when *p* will be the more common parasite genotype in the next generation, she is obviously getting a selective advantage, since her offspring will be more likely to be resistant to disease. As selection proceeds, both by the basic advantage of *H* when *p* is common and by the enhancement through any preference for *H*, the usual problem of variation damping out as all individuals become resistant might be envisioned; but meanwhile selection has been operating within the parasite population and has been favoring *P*. As the proportion of *P* individuals increases, the advantage of *H* falls; *h* then begins to increase in frequency and becomes the better genotype for females to choose.

Such a system usually has an equilibrium point where all four genotypes could occur together. But theory predicts that, given the pattern of host-parasite genotype interactions outlined above, this equilibrium point is unlikely to be stable (*6*). If it is unstable, then a limit cycle, or at least a permanently dynamical behavior of some kind, is instead the probable outcome. Cyclical selection affecting one locus in the host implies that there must be two generations per cycle where heritability is negative—those where advantage switches from *H* to *h* and vice versa. But it also implies that as cycles lengthen the *mean* parent-offspring correlation in fitness must become positive, with .5 as asymptotic upper limit. If cycles are very short, then trying to choose mates for the "right" genes for resistance is a perverse task; an animal might even do best to seek a "worst-looking" mate. Despite theoretical possibility (*7*), it is not clear yet if extremely short cycles (for example, period 2) are likely to occur in nature. Nevertheless, cycling could be relatively rapid under, in general, conditions involving intense selection pressure and pathogens that are short-lived and highly mobile and infectious (*7*). On the other hand, weak selection, approximate equality of generation time of host and parasite, and also any lag in the feedback (such as long-dormant infective eggs or a long-lived vector) tend to create long cycles. Up to a point (*8*), these should favor sexual selection.

Broadening an a priori case for cycles generally it may be noted (i) that epidemic rather than steady occurrence of disease can be involved without losing the tendency to cycle, and (ii) that existence of two or more species of parasite differently virulent to host genotypes hardly differs conceptually from the case of two or more genotypes in an asexual parasite species. In any case when several cycles

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