

Description

Technical Field

This invention relates to the field of recombinant vaccines and viral diseases. More particularly, the invention relates to use of the hepatitis A virus genomic sequence to obtain oligomeric probes for diagnosis, to obtain vaccines useful in protection of subjects at risk with respect to hepatitis A, and to obtain monoclonal antibody populations useful in treatment of infected subjects and as diagnostics.

Background Art

Hepatitis is a serious debilitating disease prevalent throughout the world. It may occur in acute form and causes extensive fatigue, liver damage, and, not infrequently, is fatal. The disease is caused by a viral infection. The infective agents are endemic in underdeveloped areas of the globe, where the majority of adults carry antibodies directed against viral antigens. In the United States, Western Europe, and Japan, the incidence of hepatitis is also significant especially among those exposed to conditions which are conducive to transmission of viral infection. However, because the probability of exposure in general is lower, most adults in these areas do not carry antibodies directed against the antigens of the virus causing hepatitis, and are at high risk when travelling to areas where the virus is endemic.

Three very different and immunologically distinct forms of virus causing hepatitis are known: Hepatitis A, Hepatitis B, and a third known at present only as Non-A/Non-B. Hepatitis B virus (HBV) has been widely studied and vaccines protective against infection by HBV have been developed. Another infective species, hepatitis A virus (HAV) is a picornavirus belonging to the enterovirus genus. HAV was first characterized as the cause of acute illness by Feinstone, S. M., et al, *Science* (1978) 182:1026-1028; Siegl, G., et al, *J Virol* - (1978) 26:40-47, 48-53. This form of the virus causes a clinically important disease, and more than 100,000 cases of HAV-induced hepatitis are reported annually in the United States. The incidence of the disease is similar elsewhere in the Western world.

As antibodies raised against hepatitis B are not cross-reactive against hepatitis A or protective against HAV infection, there is a need for the development of vaccines specifically directed against infection by HAV. While the virus has been characterized morphologically, and the genome partially sequenced, the development of a vaccine has been hindered by insufficient knowledge of the nucleotide sequence of the genome. As will be further explained below, vaccine design is dependent on identification of suitable epitopes either as primary peptide sequences, or resulting from production of 3-dimensional configurations, which engender the formation of neutralizing antibodies.

HAV has the typical icosahedral structure of a picornavirus, measuring 27-32 nm in diameter. The virions contain a single stranded genomic RNA molecule of approximately 7.5 kb, having positive strand polarity with a polyA stretch at the 3' end, and may be covalently linked to a protein at the 5' terminus. It has been assumed that the polypeptides encoded by the RNA are analogous to those of other picornaviruses, such as those responsible for polio and for foot and mouth disease (FMD). While variations occur, sufficient similarity has been shown between such viruses to warrant devising a systematic nomenclature of picornavirus proteins, as set forth in Rueckert, R. R., et al, *Journal of Virol* (1984) 50:957-959. This system of nomenclature, called the L434 convention, is based upon an idealized map of a polyprotein produced by the picornavirus genome, which can be accommodated into the pattern L-ABCD-ABC-ABCD. In this notation L is a leader protein, the first quartet represents a set of 4 capsid proteins encapsulating the viral particle, the middle triplet represents proteins of various functions, and the downstream quartet includes a proteinase capable of cleaving the polypeptide precursor of the capsid proteins into individual peptides. As shown by the invention herein, this pattern is consistent for HAV encoded polypeptides as well.

Some work relevant to vaccine preparation has been done. Monoclonal antibodies (Mabs) against HAV which are neutralizing in *in vitro* assays, but not protective against infection, were prepared by MacGregor, A., et al *J Clin Microbiol* (1983) 18:1237-1243, using spleens from mice injected with whole virus as fusion partners in preparing the Mab producing hybridomas. These Mabs appear cross-reacting, and apparently recognize antigenically similar determinants. In addition, Hughes, J. V. et al, *J Virol* (1984) 52:465-473 prepared Mabs which were capable of immunoprecipitating whole HAV, but not the disrupted or denatured proteins of the virus. These Mabs were obtained from hybridomas which had been formed using as fusion partners the spleens of mice which had been injected with a mixture of VP1, VP2, and VP3 HAV proteins. (Consistent with other picornaviruses, the capsid proteins appear to comprise three or four separate proteins, VP1, VP2, VP3, and possibly a VP4, of which VP1 is apparently the most exposed, and the major

provider of antigenic determinants.) It has been consistently shown that the whole virus contains antigenic sites which are not present in individual capsid proteins. (Gerlich, W. H., et al, Med Microbiol Immunol - (1983) 172:101-106; Hughes, J. V. et al (supra).)

Emini, E.A. et al, Abstracts; Modern Approaches to Vaccines (Sept 12-16, 1984, Cold Spring Harbor, NY) p72, have reported that poliovirus peptides corresponding to poliovirus capsid antigenic sites, when conjugated to BSA and injected into rabbits, rats or guinea pigs, were able to prime the subject to respond to sub-immunogenic administration of HAV by producing neutralizing antibodies.

Partial cloning and sequencing of the HAV genome has been reported by others (Ticehurst, J. R., et al, Proc Natl Acad Sci (USA) (1983) 80:5885-5889; Von der Helm, et al, J Virol Meth (1981) 3:37-44). These reports have yielded insufficient information to permit construction of polypeptides which can serve as vaccines. The present invention provides the entire genomic sequence of HAV, and thus permits manipulation of the potential epitopes to obtain effective vaccines against HAV.

EP-A-0154587, designating CH, DE, FR, GB, IT, LI and NL and published on 11 September 1985 discloses the partial sequence of the HAV genome. Certain fragments of this sequence are also disclosed.

EP-A-0175613, designating CH, DE, FR, GB, IT, LI and NL and published on 11 September 1985, discloses certain immunogenic peptides derived from HAV.

W085/01517 designating EPO contracting states AT, BG, CH, DE, FR, GB, LU, NL and SE and published on 11 April 1985 discloses a 1489 nucleotide sequence of HAV cDNA.

Disclosure of the Invention

The invention provides a cDNA replica of the entire HAV genomic sequence. Portions of the genomic sequence are useful as probes to diagnose the presence of virus in clinical samples. The understanding of the genomic sequence also effectively makes available the entire viral polypeptide sequence and permits not only analysis, but production of portions thereof, if appropriate, in the correct configuration, so as to provide effective vaccines and permit the production of neutralizing antibodies. The availability of the entire HAV sequence thus permits the design and construction of polypeptides which may serve either as vaccines themselves, or as intermediates in the production of monoclonal antibody (Mab) preparations useful in passive immunotherapy against the disease, or as diagnostic reagents. Without the sequence of the entire genome at the disposal of the producer of the therapeutic or preventive compositions, successful production of optimally effective products would be impossible.

Accordingly, in one aspect, the invention relates to a nucleotide sequence substantially identical with that representing the entire genome of HAV as represented in Figure 1. The remaining contributions of the invention relate to utilizing this sequence or portions thereof as oligomeric probes, to produce peptides which can serve as vaccines, or as reagents to produce monoclonal antibodies useful in treatment of the disease.

Other aspects of the invention include expression systems which are capable of effecting the production of a desired protein encoded by sequences derived from the complete genome, to recombinant vectors containing such systems or portions thereof, to recombinant host cells transformed with such vectors, to proteins produced by the transformed cells, and to vaccines prepared from such proteins. In addition, the invention relates to specific peptide sequences representing epitopes encoded by the genome, and to such sequences covalently linked to carrier proteins. Such carrier proteins, in addition to more conventional carriers, include the 22 nm particle associated with hepatitis B infection, which carries polyalbumin receptor sites, and is 1000 fold more immunogenic than the unassembled subunit component. By inserting antigenic HAV determinants into the 22nm HBsAg particle, increased immunogenicity for these epitopes is obtained. The invention also relates to deleted virions capable of eliciting neutralizing antibodies in subject hosts, and to monoclonal antibodies useful in passive immunotherapy.

The invention also relates to the methods of preparing these desired polypeptide vaccines and immunoglobulins.

Insofar as the invention relates to portions of the sequence of Figure 1 and their use in expression vectors or as oligonucleotide probes, those portions of the sequences are those claimed in claim 3.

Brief Description of the Drawings

Figure 1 shows the complete nucleotide sequence of the HAV genome. The cDNA contains the identical sequence, except, of course, that T will be substituted for U. The deduced amino acid sequence of the viral L434 polypeptide is also shown in Figure 1.

Figure 2 shows a restriction map and the regions of overlap of the four cDNA clones which comprise

the entire 7.5 kb HAV genome, pHAV47, pHAV8, pHAV1, and pHAV16.

Figure 3 shows the construction of expression vectors for sequences encoding fusion mRNAs encoding portions of superoxide dismutase (SOD) and HAV capsid protein.

Figure 4 shows a computer generated profile based on hydrophilicity and structural characteristics of the peptide chain as a function of position for regions of the polio virus polypeptide and the HAV polypeptide.

Figure 5 shows the construction of a mammalian cell expression vector encoding deleted HAV virions.

Figure 6 shows the construction of pDC-104 for production of hybrid HAV/HBsAg in yeast.

Figure 7 shows the construction of pWG-1 for production of hybrid HAV/HBsAg in yeast.

Modes of Carrying Out the Invention

A. Definitions

As used herein, a nucleotide sequence "substantially identical" to the exemplified HAV genome refers to a sequence which retains the essential properties of the exemplified polynucleotide. A specific, but non-limiting example of such substantial equivalence would be represented by a sequence which encodes the identical or substantially identical amino acid sequence, but, because of codon degeneracy, utilizes different specific codons. Nucleotide changes are, indeed, often desirable to create or delete restriction sites, provide processing sites, or to alter the amino acid sequence in ways which do not adversely affect functionality. "Nucleotide sequence" refers both to a ribonucleotide and a deoxyribonucleotide sequence and includes the positive sense strand, as shown, and the negative sense strand as well.

A DNA sequence "derived from" the nucleotide sequence which comprises the genome of HAV refers to a DNA sequence which is comprised of a region of the genomic nucleotide sequence, or a combination of regions of the sequence. These regions are, of course, not necessarily physically derived from the nucleotide sequence of the gene, but refer to polynucleotides generated in whatever manner which have the same or "substantially identical" sequence of bases as that in the region(s) from which the polynucleotide is derived. For example, typical DNA sequences "derived from" the HAV genome include fragments encoding specific epitopes, fragments encoding portions of the viral polypeptide, sequences encoding the capsid proteins, sequences encoding deleted virions, and sequences encoding the viral proteinase. Similarly, a peptide "derived from" the HAV polypeptide refers to an amino acid sequence substantially identical to that of this polypeptide or a portion thereof, having the same biological properties as that portion. The manner of synthesis of such "derived" peptide is not material—it may be chemical synthesis or recombinant means, for example.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eucaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of the nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

"Control sequence" refers to DNA sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending on the host organism; in procaryotes, generally such control sequences include promoter and ribosome binding site; in eucaryotes, generally, such control sequences include promoter, terminators, and, in some instances, enhancers. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

B. General Description

At the core of the present invention is the provision of a nucleotide sequence containing the entire

genome of hepatitis A virus. The availability of this complete polynucleotide, first, permits the construction of oligomers of about 20 bp or more useful in disease diagnosis. Such probes may be used to detect the presence of the viral genome in, for example, fecal filtrates of subjects suspected of harboring the virus. Knowledge of the gene sequence also enables the design and production of vaccines effective against HAV and production of neutralizing Mabs. Sequencing information available from the genome allows the amino acid sequence of the polypeptide to be deduced and compared to that of other known picornaviruses for which locations of favorable epitopes is known. Further, once the desired sequences are chosen, fragments of the genome can be obtained and expressed independently, thus providing desired polypeptides using recombinant techniques. Both procaryotic and eucaryotic hosts are useful for such expression. Short polypeptide fragments may be chemically synthesized and linked to carrier proteins for use as vaccines. Recombinantly expressed polypeptides may be provided under conditions offering a favorable environment for processing into, for example, deleted virions, thus permitting three dimensional reconstruction of epitopic sites without danger of creating an infective virus. Mammalian and yeast cells provide particularly suitable environments for such expression. In addition, the epitopes may be produced linked to a particle forming protein. The proteins thus produced may, themselves be used as vaccines, or may be used to induce immunocompetent B cells in hosts; which B cells can then be used to produce hybridomas that secrete antibodies useful in passive immunotherapy.

B.1. Preparation of the HAV Gene Sequence

The genomic sequence of HAV was prepared using stool samples of infected patients as the source for the virus. The isolated viral RNA was fractionated, and those fractions containing RNA of sufficient length to contain the entire genome were pooled, ethanol precipitated, and used to obtain a cDNA library. Colonies from the library were hybridized to oligomeric probes complementary to the 3'-proximal portions of the RNA. Several colonies hybridizing to these sequences were used to obtain additional portions of the RNA message from the cDNA library using "walking" techniques. Four cDNA clones were obtained from overlapping portions of the gene, and subjected to restriction mapping and sequencing. The entire genomic sequence was deduced from these four cDNA inserts. The amino acid sequence deduced from the viral genome is similar to that characteristic of picornaviruses, and the 5' and 3' non-coding regions probably involved in viral replication were identified.

B.2. Preparation of Viral Polypeptide Fragments in *E. coli*

The availability of the entire genomic sequence permits construction of expression vectors encoding presumptively antigenically active regions of the capsid proteins. Fragments encoding the desired proteins are obtained from the cDNA clones using conventional restriction digestion and ligated into vectors containing portions of fusion sequences such as β -galactosidase or SOD, preferably SOD. Any desired portion of the HAV genome could be expressed as a fusion protein or co-expressed with another readily produced protein in *E. coli* using this approach. However, for the purpose of generating peptides capable of raising antibodies against HAV, fragments representing portions of the genome encoding the capsid protein regions VP1, VP2 and VP3 were used. In a particularly preferred mode, human SOD encoding sequences are fused to those derived from HAV as described in more detail hereinbelow. Alternatively, since a three-dimensional antigenic site may result from suitable arrangement of individual peptide chains, bacterial expression vectors including the coding sequence for the viral proteinase, which would be encoded in the downstream quartet, may be included on the same, or cotransformed expression vector as that constructed for the precursor (PI) of the capsid proteins. Simultaneous expression of the capsid encoding and proteinase encoding sequences may also result in preparations which produce the various capsid proteins as individual peptides. (See, e.g., Klump, W., et al, *Proc Natl Acad Sci (USA)* (1984) 81:3351-3355; Hanecak, R., et al, *Cell* (1984) 37:1063-1073.) These may then assemble to the appropriate three-dimensional structure.

B.3. Preparation of Antigenic Polypeptides and Conjugation with Carrier

The availability of the entire genomic sequence also makes possible computer analysis of the structural characteristics of the deduced protein for comparison with the known sequence of polio virus. As the antigenic regions of polio virus are known, such comparisons make possible the designation of short peptide regions representing epitopes. These can then be synthesized using chemical methods, and provided with, for example, cysteine residues at the C terminus which provide means for linking the

peptides to neutral carrier proteins. A number of techniques for obtaining such linkage are known in the art, including the formation of disulfide linkages using N-succinimidyl-3-(2-pyridylthio)-propionate (SPDP) and succinimidyl 4-(N-maleimido-methyl) cyclohexane-1-carboxylate (SMCC) obtained from Pierce Company, Rockford, Illinois. These reagents create a disulfide linkage between themselves and peptide cysteine residues on one protein and an amide linkage through the ϵ -amino on a lysine, or other free amino group in the other. A variety of such disulfide/amide-forming agents are known. See, for example, *Immun. Rev.* - (1982) 62:185. Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thioether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid 2-bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl) cyclohexane-1-carboxylic acid, and the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt. The foregoing list is not meant to be exhaustive, and modifications of the named compounds can clearly be used.

Any carrier may be used, which does not itself induce the production of antibodies harmful to the host, such as the various serum albumins, tetanus toxoids, or keyhole limpet hemocyanin (KLH).

The conjugates, when injected into suitable subjects, will result in the production of antisera which contain immunoglobins specifically reactive against not only the conjugates, but also against fusion proteins carrying the analogous portions of the sequence, and against appropriate determinants within whole HAV.

B.4. Preparation of Non-Infectious (Deleted) Virions in Prokaryotes or Eucaryotes

Virions which mimic the three-dimensional epitopic configuration of intact HAV may also be prepared. It is generally surmised that antigenic sites may result from three-dimensional folding of immunogenic proteins, and, specifically with respect to HAV, it has been reported that antibodies formed in response to injection with HAV capsid proteins are not reactive against the denatured viral peptides (Gerlich et al, *Med Microbiol Immunol* (1983) 172:101-106).

The deleted virions are prepared by providing DNA sequences which lack the 5' and 3' non-coding regions of HAV putatively responsible for replicative functions. The sequences also lack a portion of the 3' coding region presumed, by homology with other picornaviruses, to encode the replicase origin. These fragments are preferably cloned into plasmid expression vectors appropriate for yeast or mammalian cells capable of the desired post-transcription and post-translation processing; however, prokaryotes can also be used. Deleted virions produced in hosts transformed with these vectors are immunoreactive against human convalescent HAV antisera, and can be used for immunization.

B.5. Preparation of Hybrid Particle Immunogens Containing HAV Epitopes

The immunogenicity of the epitopes of HAV may also be enhanced by preparing them in mammalian or yeast systems fused with particle-forming proteins such as that associated with hepatitis B surface antigen. Constructs wherein the HAV epitope is linked directly to the particle-forming protein coding sequences produce hybrids which are immunogenic with respect to the HAV epitope. In addition, all the vectors prepared include epitopes specific to hepatitis B virus (HBV), having various degrees of immunogenicity, such as, for example, the pre-S peptide. Thus, particles constructed from particle-forming protein which include HAV sequences are immunogenic with respect to both HAV and HBV.

Hepatitis surface antigen (HBsAg) has been shown to be formed and assembled in *S. cerevisiae* - (Valenzuela et al, *Nature* (1982) 298:344-350), as well as in, for example, mammalian cells (Valenzuela, P., et al, *Hepatitis B* (1984), Millman, I., et al, ed, Plenum Press, pp. 225-236). The formation of such particles has been shown to enhance the immunogenicity of the monomer subunit. The constructs may also include the immunodominant epitope of HBsAg, comprising the 55 amino acids of the presurface (pre-S) region. (Neurath et al, *Science* (1984) 224:392-394.) Constructs of the pre-S-HBsAg particle expressible in yeast are disclosed in U.S. Serial No. 621,756, filed 18 June 1984; hybrids including heterologous viral sequences for yeast expression are disclosed in U.S. Serial No. 650,323, filed 13 September 1984. Both applications are assigned to the herein assignee and incorporated by reference. These constructs may also be expressed in mammalian cells such as Chinese hamster ovary cells using an SV40-dihydrofolate reductase vector (Michelle et al, *Int Symp on Viral Hepatitis* (1984)).

In addition, portions of the particle-forming protein coding sequences per se may be replaced with codons for an HAV epitope. In this replacement, regions which are not required to mediate the aggregation of units to form immunogenic particles in yeast or mammals can be deleted, thus eliminating additional hepatitis B antigenic sites from competition with the HAV epitope.

B.6. Preparation of Vaccines

Preparation of vaccines which contain peptide sequences as active ingredients is also well understood in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified or the protein encapsulated in liposomes. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkaline glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of manitol, lactose, starch magnesium stearate, sodium saccharine cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each subject.

B.7. Preparation of Mabs Against HAV Epitopes

The immunogenic proteins prepared as described above are used to immunize mammals. Lymphocytes from these animals may be used to prepare hybridomas capable of secreting monoclonal antibodies directed against these epitopes and cross-reactive against the infective virus. The resulting monoclonal antibodies are particularly useful in diagnosis, and those which are neutralizing are useful in passive immunotherapy.

C. General Methods

The general techniques used in extracting RNA from the virus, preparing and probing a cDNA library, sequencing clones, constructing expression vectors, transforming cells, and the like are known in the art and laboratory manuals are available describing these techniques. However, as a general guide, the following sets forth some sources currently available for such procedures, and for materials useful in carrying them out.

C.1. Hosts and Expression Control Sequences

Both procaryotic and eucaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences are used compatible with the designated host. Among procaryotic hosts, *E. coli* is most frequently used, mostly for convenience. Expression control sequences for procaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with procaryotic hosts are commonly derived from, for example, pBR322 a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance. The foregoing operons may be used as markers to obtain successful transformants by selection. Commonly used procaryotic control sequences include the β

lactamase (penicillinase) and lactose promoter systems (Chang, et al, *Nature* (1977) 198:1056, the tryptophan (trp) promoter system (Goeddel, et al, *Nucleic Acids Res* (1980) 8:4057) and the λ derived P_L promoter and N gene ribosome binding site (Schimatake, et al, *Nature* (1981) 292:128) and the hybrid tac promoter (De Boer, et al, *Proc Natl Acad Sci (USA)* (1983) 80:21-25) derived from sequences of the trp and the lac UV5 promoters. The foregoing systems are particularly compatible with *E. coli*; if desired other procaryotic hosts such as strains of *Bacillus* or *Pseudomonas* may be used, with corresponding control sequences.

Eucaryotic hosts include yeast and mammalian cell culture. *Saccharomyces cerevisiae*, or Baker's yeast and *Saccharomyces carlsbergensis* are the most commonly used yeast hosts, again because of convenience. Yeast compatible vectors carry markers which permit selection of successful transformants by conferring prototrophy to auxotrophic mutants or by conferring antibiotic resistance or resistance to heavy metals on wild-type strains. Yeast compatible vectors may employ the 2 micron origin of replication (Broach, J., et al, *Meth Enz* (1983) 101:307) the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences which will result in incorporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors include promoters for the synthesis for glycolytic enzymes (Hess, et. al, *J Adv Enzyme Reg* (1968) 7:149, Holland, et al, *Biochemistry* (1978) 17:4900), and the promoter for 3 phosphoglycerate kinase (Hitzeman, et al, *J Biol Chem* (1980) 255:2073). For yeast expression, terminators may also be included, such as those derived from the enolase gene (Holland, M. J., *J Biol Chem* (1981) 256:1385). Particularly useful control systems include those specifically described herein, which comprise the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, terminators also derived from GAPDH, and, if secretion is desired, leader sequence from yeast alpha factor. These systems are described in detail in U.S. Serial Nos. 468,589 and 522,909, filed 22 February 1983 and 12 August 1983, respectively, both assigned to the herein assignee, and incorporated herein by reference.

Mammalian cell lines available as hosts for expression include many immortalized cell lines available from the American Type Culture Collection, including HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and a number of other cell lines. Suitable promoters for mammalian cells prominently include viral promoters such as that from Simian virus 40 (SV40) (Fiers, et al, *Nature* (1978) 273:113 or other viral promoters such as the Rous sarcoma virus (RSV) adenovirus, and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences. Vectors suitable for replication in mammalian cells may include viral replicons, or sequences which insure integration of the appropriate sequences into the host genome.

C.2. Transformations

The transformation procedure used depends on the host to be transformed. Bacterial transformation generally employs treatment with calcium or rubidium chloride (Cohen, S. N., *Proc Natl Acad Sci (USA)* (1972) 69:2110, Maniatis, et al, *Molecular Cloning: A Laboratory Manual* (1982) Cold Spring Harbor Press, page 254). Yeast transformations may be carried out using the method of Hinnen, et al; *Proc Natl Acad Sci* (1978) 75:1929-1933. Mammalian transformations are conducted using the calcium phosphate precipitation method of Graham and van der Eb, *Virology* (1978) 52:546, or the various modifications thereof.

C.3. Vector Construction

Vector construction employs techniques which are by now quite well understood. Site-specific DNA cleavage is performed by treating with suitable restriction enzyme under conditions which generally are specified by the manufacturer of these commercially available enzymes (see, e.g., The New England Biolabs Product Catalog). In general, about 1 μ g of plasmid or DNA sequence is cleaved by 1 unit enzyme in about 20 μ l buffer solution for an incubation time of about 1-2 hr at about 37° C. After incubation with the restriction enzyme, protein is removed by phenol/chloroform extraction and the DNA recovered by reprecipitation with ethanol. The cleaved fragments may be separated using polyacrylimide or agarose gel electrophoresis techniques, according to the general procedures found in *Methods in Enzymology* (1980) 65:499-560.

Sticky ended cleavage fragments may be blunt ended using *E. coli* DNA polymerase I (Klenow) in the presence of the appropriate deoxynucleotide triphosphates (dNTPs) using incubation conditions appropriate to the polymerase. The polymerase digests protruding 3' single strands, but fills in 5' protruding ends, according to the dNTPs present in the mixture. Treatment with S1 nuclease may also be used, as this results in hydrolysis of any single stranded DNA portion.

Ligations are carried out using standard buffer and temperature conditions using T4 DNA ligase, and ATP; sticky end ligations require less ATP and less ligase than blunt end ligations. When vector fragments are used as part of a ligation mixture, the vector fragment is often treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and thus prevent religation of the vector; alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent religation.

Ligation mixtures are transformed into suitable cloning hosts, such as *E. coli*, and successful transformants selected by, for example, antibiotic resistance, and screened for the correct construction.

C.4. Construction of Desired DNA Sequences

Synthetic oligonucleotides may be prepared using an automated oligonucleotide synthesizer as described by Warner, B.D., et al, *DNA* (1984)3:401-411. If desired, these synthetic strands may be kinased for labeling with ³²P by using an excess of polynucleotide kinase in the presence of labeled ATP, under standard kinasing conditions.

DNA sequences including those isolated from genomic or cDNA libraries may be modified by site directed mutagenesis, as described by Zoller, M, et al, *Nucleic Acids Res* (1982) 10:6487-6499. Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified, and having the desired modification included in its own sequence. The resulting double stranded DNA is transformed into a phage supporting host bacterium, and cultures of the transformed bacteria, which will contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically 50% of the new plaques will contain phage having as a single strand the mutated form: 50% will have the original sequence. Replicates of the plaques are hybridized to kinased synthetic probe at temperatures and conditions which permit hybridization with the correct strand, but not with the unmodified sequence. The thus identified, desired, modified sequences are then recovered and cloned to serve as sources for the desired DNA.

C.5. Hybridization with Probe

DNA libraries are probed using the procedure of Grunstein and Hogness (*Proc Natl Acad Sci (USA)* (1975)73:3961). Briefly, in this procedure, the DNA to be probed is immobilized on nitrocellulose filters, denatured, and prehybridized with a buffer containing 0-50% formamide, 0.6 M NaCl, 60 mM sodium citrate, 0.02% (wt/v) each of bovine serum albumin, polyvinyl pyrrolidone, and Ficoll, 50 mM sodium phosphate (pH 6.5), 1% glycerine, and 100 µg/ml carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the prehybridization and subsequent hybridization steps depends on the stringency desired. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and longer hybridization times. Probes containing more than 30 or 40 nucleotides such as those derived from cDNA or genomic sequences generally employ higher temperatures, e.g. about 40-42° and a high percentage, e.g. 50% formamide. Following prehybridization this same buffer, now containing the ³²P kinased oligonucleotide probe, is added to obtain hybridization. Radioautography of the treated filters shows the location of the hybridized probe, and the corresponding locations on replica filters which have not been probed can then be used as the source of the desired DNA.

C.6. Verification of Construction and Sequencing

For routine vector constructions, ligation mixtures are transformed into *E. coli* strain HB101 or other suitable host, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al, *Proc Natl Acad Sci (USA)* (1969) 62:1159, usually following chloramphenicol amplification (Clewell, D. B., *J Bacteriol* (1972) 110:667). The isolated DNA is isolated and analyzed by restriction analysis, or sequenced by the dideoxy method of Sanger, F., et al, *Proc Natl Acad Sci (USA)* (1977) 74:5463, as further described by Messing, et al, *Nucleic Acids Res* (1981) 9:309, or by the method of Maxam, et al, *Methods in Enzymology* (1980) 65:499.

D. Examples

The following examples are intended to illustrate but not limit the invention. The procedures set forth,

for example, in 1D.1 may, if desired, be repeated but need not be, as techniques are available for construction of the desired nucleotide sequences based on the information provided by the invention. Expression is exemplified in *E. coli* and in yeast, however other systems are available as set forth more fully in 1C.1. Additional epitopes derived from the genomic structure may also be produced, and used to generate antibodies as set forth below.

D.1. Preparation of HAV cDNA

HAV virus was isolated from the stools of a hepatitis A patient collected during an epidemic outbreak in Los Angeles, California. Other isolates, available in the art could also have been used. See, for example, Daemer, R. J., et al, *Infect Immunity* (1981) 32:388-393; Frosner, G. G., et al, *Infection*, (1979) 7:303-350. Techniques for such isolation, and propagation are well known in the art.

The isolate was inoculated onto PCL/PRF/5 (Alexander hepatoma cells) followed by culture at 34°C. Detectable virus production was observed 3-4 weeks later, and the original infected cell cultures have been continuously passaged in DME/15% FCS for the past two years with no loss of virus titers. (Virus titers were measured by immunofluorescence and/or competitive radio immunoassay (HAVAB, Abbott).)

Viral RNA was extracted from the virions by conventional procedures, for example, that disclosed by Ticehurst, J. E., et al, *Proc Acad Sci (USA)* (1983) 80:5885-5889, including protease treatment and phenyl/chloroform extraction, followed by ethanol precipitation. Low molecular weight contaminants were fractionated from genome length RNA by 15-30% sucrose gradients run for 5 hr at 40,000 x g in a SW 41 Spinco rotor. Gradients were collected onto 22-24 fractions, each fraction precipitated, and run on a formaldehyde-agarose gel (Lehrach, H, et al, *J. Biochem* (1977) 16:474c. Each gel was transferred to a nitrocellulose membrane and hybridized to labeled specific probe as described by Thomas, *Proc Natl Acad Sci (USA)* (1980) 77:5201.

Prehybridization and hybridization were essentially as described above. Probes were kinased at the 5' terminus with T4 kinase according to the method of Lillehaug, et al, *Biochemistry* (1976) 15:1858 followed by purification on a Sep-pak (Millipore) filter. Two oligonucleotide probes, designated HAV 1 and 2 were constructed based on sequencing information obtained by Ticehurst, et al, (supra). The probes had the following sequences:

HAV1: 3'-ACAAATAAAGAAAATAGTCATTTA-5'

HAV2: 3'-ATGTCTGAATTTAGAACTAACCACC-5'

These probes were thus validated by hybridization to the gels run on HAV RNA from partially purified virions.

RNA from the sucrose fractions which contain full length HAV RNA were identified by the above-described hybridization blotting techniques. Samples were pooled, ethanol precipitated and used to form a cDNA library. The cloning procedure used is a modification of that previously described by Wood and Lee, *Nucleic Acid Res* (1976) 3:1961; Lain et al, *Cell* (1979) 16:851. The two modifications introduced with respect to the published procedures are as follows: (a) a short double-stranded synthetic primer dT was annealed with the poly A+ RNA to synthesize the cDNA; (b) after dC tailing, the cDNA:RNA hybrid molecules were then annealed with PstI-cleaved dG-tailed pBR322. Before the transformation, the pBR322:mRNA:cDNA molecules were repaired with concentrated extract of activated *Xenopus* eggs which was prepared as indicated below. The ligation mixtures were then transformed into *E. coli* K12 MC1061 to Amp^R.

Xenopus laevis eggs were obtained as described by Newport and Kirschner, *Cell* (1982) 30:675-686, and were washed twice with MMR (0.1 M NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM Hepes, pH 7.8, 0.1 mM EDTA) and dejellied in 2% cysteine. The eggs were then washed twice with MMR/4 and activated by electric shock (5 sec, 12 V); incubated 3 hours at room temperature, followed by 2 washed with 160 mM β-glycerophosphate, 2 mM EGTA, 10 mM MgCl₂, 10% glycerol, 1 mM PMSF, 25 μg/ml creatine-phosphokinase, 5 mM phosphocreatine, pH 7.5; and then crushed by pipetting and centrifuged for 10 minutes at 10,000 rpm. The crude extract was collected through the lipid layer and recentrifuged for 30 minutes at 40,000 rpm. The clear extract was aliquoted and stored frozen at -80°C.

The resulting cDNA library was probed by transferring the colonies to replicate nitrocellulose filters, and hybridizing to HAV1 and HAV2 as described above. Using the foregoing probes, and selecting colonies hybridizing to both, 3' proximal clones were obtained. Further portions of the genome were identified by "walking" techniques. An additional clone was obtained using the 5' terminal PstI/PstI fragment from pHAV1307 as probe (Ticehurst, J., et al, (Supra)).

Four recombinant cDNA inserts designated pHAV16, pHAV1, pHAV8, and pHAV47 were selected and restriction maps prepared. The overlapping regions were confirmed by cross-hybridization studies, and the

total length of non-overlapping regions was approximately 7.5 kb, the entire span of the HAV genome. The results of the restriction mapping and overlap are shown in Figure 2. pHAV16, pHAV1, pHAV8 and pHAV47 were deposited with the American Type Culture Collection (ATCC) on 29 March 1985 and assigned accession numbers 53077, 53078, 53076 and 53079, respectively.

Complete sequencing of the series of four inserts shown in Figure 2 gave the results for the corresponding viral RNA set forth in Figure 1. (Analysis of the open reading frames associated with this sequence indicated a deletion of 22 nucleotides at position 3530 of the HAV genome; this was corrected by site-directed metagenesis to obtain the sequence shown in Figure 1; the corrected plasmid was designated pHAV1*.)

The 7,478 nucleotide heteropolymeric sequence is followed by a polyA tract of undetermined length. A single open reading frame starts with an AUG triplet at nucleotide 734 and terminates with a UGA codon at nucleotide 7,415. Thus, the polyprotein encoded is 2227 amino acids and has a molecular weight of 251,940 daltons. The deduced genomic RNA sequence in Figure 1 shows the 5' non-coding region required for linkage to a tyrosine residue on a viral protein, and the first 75 nucleotides of the sequence can be arranged in the highly stable secondary structure comprising stems and loops known from other picornavirus RNA sequences. Also, the 5' terminal portion contains long U+C repeats, as is generally observed in other picornaviruses.

D.2. Construction of Expression Vectors and Expression of HAV Sequences in E. coli

Four bacterial expression plasmids were constructed which directed the simultaneous synthesis of human superoxide dismutase (SOD) (Hallewell, et al, *Nucleic Acids Res* (1985) 13:2017 and also portions of all of the hepatitis A capsid precursor protein, P1 (Najarian, et al, *Proc Natl Acad Sci (USA)* (1985) 82:2627. Three of the plasmids synthesized part or all of the P1 fused to the carboxy terminus of SOD. The fourth plasmid also generated a fused mRNA for SOD and P1 gene sequences but contained a sequence between the SOD and P1 genes which provided a stop codon for translation termination of SOD along with a ribosome binding site for reinitiation of translation at the methionine codon thought to be the natural initiator codon of P1. The constructions are shown schematically in Figure 3.

The expression plasmids were based on the tac promoter driven expression plasmid pSOD16 of Hallewell, et al, (supra). Plasmid pSOD16cf2 was generated from pSOD16 by replacement of a portion of the carboxy terminal coding region of the SOD gene and downstream polylinker sequence through the EcoRI site by the new polylinker sequence

5' GATCGCCATGGGTACCCGGGTCGACTAAATGACTAG 3'
3' CGGTACCCATGGGCCAGCTGATTTACTGATCTTAA 5'

The substitution of this polylinker sequence results in the removal of the natural carboxy terminal Gln of SOD.

pSODHAV2-2

A 1390 base pair BglII(repair)/XmnI fragment isolated from a digest of pHAV8116SV7d (see ¶ D.4) was cloned into the SmaI site of pSOD16cf2. DNA of a resulting clone having the HAV insert in the proper orientation was digested with KpnI, the 3' overhang was removed with T4 DNA polymerase, then digested the HindIII, and this site was filled in with reverse transcriptase. The resultant linear, blunt ended DNA was religated and transformed into *E. coli* strain D1210 (Salder, et al, *Gene* (1980) 8:279-300) to obtain pSODHAV2-2.

pSODHAV2-2 was transformed into *E. coli* RRIAM15 (Ruther, U., *Nucleic Acids Res* (1982) 10:5765-5772) for expression to obtain a fusion protein of SOD with the natural SOD carboxy terminal Gln replaced by Met-Glu followed by HAV sequence beginning with the Leu encoded at HAV nucleotide 2072 of the VP3 capsid protein and extending through VP1 to the Val encoded at nucleotide 3201 and terminated with the sequence Gly-Arg-Leu-Asn- Asp encoded by the polylinker.

pSODHAVP1/1

pSODHAVP1/1 contains, in addition to the sequences in pSODHAV2-2, the remainder of VP3 and the

carboxy half of VP2. pSODHAV2-2 was digested with NcoI (5' to the HAV sequences) and with SacI, which cuts within the VP1 sequence. The 3.4 kb fragment containing all the SOD sequences and a part of the VP1 sequences was gel isolated, ligated to a 1806 bp NcoI/SacI fragment isolated from pHAV81167d (see ¶ D.4) and transformed into *E. coli* strain D1210 to obtain pSODHAVP1/1.

When expressed in *E. coli* strain RRIAM15, SODHAVP1/1 generated a fusion protein of SOD with HAV sequences starting at the Val of VP2 encoded at nucleotide 1182 through Val of VP1 encoded at nucleotide 3201.

pSODHAV9-1

pSODHAV9-1 was derived from pSODHAVP1/1 and contains the additional half of VP2 to generate the full P1 precursor sequence. A 438 bp BstXI fragment from PHAV47 (see ¶ D.1) containing the 5' end of VP2 was gel isolated and ligated to the single stranded synthetic DNA oligomer,

5'-ATGAATATGTCCAAACAAGGAATTTTCCAGACTG-3'

via the BstXI overhang at the 3' end of the 438 bp fragment. The single stranded sequence corresponds to the coding strand of the HAV genome from the extreme 5' end of the P1 precursor. The oligomer was phosphorylated at its 5' end and the strand complementary to the oligomer was synthesized with reverse transcriptase using the 3' hydroxyl of the BstXI overhang of the 438 bp fragment as primer. The resulting fragment was then digested with NcoI, and the NcoI/blunt fragment including the added 30 base pairs of double stranded oligomer, was gel isolated and ligated to pSODHAVP1/1, which had been previously digested with NcoI, at room temperature for 2 hr. The reaction was diluted, and the four dNTPs, Klenow, and additional ligase added to circularize the DNA. The ligation mixture was transformed into *E. coli* RRIAM15 to obtain pSODHAV9-1 which contains HAV nucleotides 734 to 3201 (VP2, VP3, VP1).

pSODHAV10-5

pSODHAV10-5 was constructed in a manner analogous to that described above for pSODHAV9-1 except that the synthetic DNA oligomer

5' GATAGAGGAATTATAAGATGAATATGTCCAAACAAGGAATTTTCCAGACTG 3'

was used. The oligomer provides for stop codon and a ribosome binding site, in addition to HAV sequences, downstream. pSODHAV10-5 when expressed results in free SOD with the natural terminal Glu replaced with the sequence Met-Ile-Glu-Glu-Leu, and also free HAV P1 lacking its 13 carboxy terminal amino acids, which were replaced with Gly-Arg-Leu-Asn-Asp.

Expression

E. coli strain RRIAM15, Ruther, U. (supra), cells were transformed with the plasmids described above and single colony transformants were grown overnight at 37°C in 2 ml L-broth plus 100 µg/ml ampicillin. Glycerol (50%) stocks of these cultures were prepared and stored at -20°C.

For protein expression analysis, overnight cultures, in medium as above, were begun from glycerol stocks. These cultures were diluted 1/100 into the same medium and grown at 37°C to an OD₆₅₀ of 0.5-0.8. Uninduced controls were prepared by diluting a 1 ml portion of the growing culture with an equal volume of the same medium. Tac promoter-driven protein synthesis was induced either by diluting of 1 ml of growing culture with medium plus 2 mM IPTG (isopropyl β-thiogalactopyranoside) or by addition of 1/100 volume of 100 mM IPTG to larger volume cultures, in either case, to give 1 mM IPTG, for 3-4 hr at 37°C. Large scale cultures had 50 ml medium in 500 ml flasks or 250 ml medium in liter flasks.

Cells were lysed in the presence of SDS and DTT for analysis on denaturing polyacrylamide gels (Leammli, *Nature* (1970) 277:680) or with glass beads for analysis on non-denaturing polyacrylamide gels.

Expression was detected by the appearance of (a) protein band(s) of expected size present in induced sample lysates but not in uninduced samples; induced proteins were detected at their respective, expected molecular weights for all plasmids.

The HAV component of fusions and the free P1 of pSODHAV10-5 were detected by Coomassie staining and by specific reaction with anti-HAV antibodies. The SOD component of fusions was detected by Coomassie staining and by anti-SOD antibodies; free SOD was detected both by Coomassie stain and by

enzymatic activity in native gels. Gels were either stained with Coomassie blue or subjected to Western analysis after electroblotting onto nitrocellulose filters (Towbin et al, Proc Natl Acad Sci (USA) (1979) 76:3450); blots were preincubated with 0.3% Tween 20 in PBS for 1 hr at room temperature or overnight at 4°C, treated with anti-HAV or anti SOD at 1:100 dilution, and incubated for 1 hr at room temperature. (Rabbit anti-HAV was prepared using partially disrupted HAV virions. Anti-human SOD was prepared by sequential exposure of rabbits to purified recombinant human SOD produced in yeast (see EPO 138 111).) Blots were washed extensively in 0.3% Tween/PBS and incubated for 1 hr at room temperature with a 1:200 dilution of horseradish peroxidase conjugated goat anti-rabbit-IgG antibody, then washed and treated with Bio-Rad HRP color reagent at 0.5 mg/ml in 8 mM Tris HCl pH 7.5, 16 mM NaCl, 0.015% H₂O₂, 16% methanol.

SOD activity in non-denaturing gels was identified by the method of Beauchamp, C., et al, Anal Biochem (1971) 44:276. Briefly, gels were soaked in the absence of light, first in 2.5 mM nitro blue tetrazolium for 20 min at room temperature, then in 36 mM KPO₄, pH 7.8, 29 mM TEMED, 28 µM riboflavin for 15 min at room temperature. Buffer was removed and the gel was illuminated for 15 min from below using a fluorescent light box. The results of these analyses are shown in Table 1.

Table 1

Plasmid	MW (induced)		Coomassie staining (PAGE)	Western with anti-HAV antibody	SOD activity
	Calculated	Observed			
pSODJAV2-2	59,120	58,880	+	+	nt
pSODHAVP1/1	92,160	88,820	+	+	-
pSODHAV9-1	108,910	105,900	-	+	nt
pSODHAV10-5	92,970 (P1)	84,140	-	+	-
	16,440 (SOD)	20,650	+	-	+

nt=not tested

Expressed products were approximately 1-3% of total cell protein for pSODHAV2-2 and pSODHAV1/1 and about 50-fold lower for pSODHAV9-1 and pSODHAV10-5.

The SODHAV2-2 and SODHAVP1/1 fusion proteins were purified on preparatory SDS-acrylamide gels, located by Coomassie staining, and electroeluted. These purified proteins were injected into mice and the resulting sera were tested for reactivity with HAV virus on Western blots and in ELISA assays. Mouse antisera against the SODHAV2-2 react with a 34 kd protein from HAV virus on Western blocks that appears to be HAV-VP1 and was therefore also designated anti-SOD-VP1. In a capture ELISA both mouse anti-SODHAV2-2 and anti-SODHAV1/1 react with HAV virus.

In the ELISA assay, microliter wells were coated overnight with IgG from an HAV seropositive patient. The coated wells were washed and incubated 1 hr with purified HAV. Dilutions of the mouse sera were the incubated in the well for 1 hr. The wells were then incubated 1 hr with peroxidase conjugated goat anti-mouse Ig followed by reaction with the peroxidase substrate 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) and H₂O₂, and the plates were read on an ELISA reader at 414 nm; the titer is expressed as the reciprocal of the dilution that gives 50% of the maximum absorbance. The results are as follows:

	Control Mouse a	< 10
	Control Mouse b	< 10
5	Mouse Anti-SOD-VP1 a	308
	Mouse Anti-SOD-VP1 b	158
	Mouse Anti-SOD-P1 a	183
	Mouse Anti-SOD-P1 b	160
10	Mouse Anti-SOD-P1 c	30
	Mouse Anti-SOD-P1 d	42

15 In additional assays, 20 ng of SODHAV2-2 and SODHAVP1/1 were spotted onto nitrocellulose strips, and the strips were then reacted with two HAV seropositive human sera and two seronegative human sera. The strips were washed and reacted with peroxidase conjugated goat anti-human IgG, and then reacted with the peroxidase substrate 4-chloro-1-naphthol to visualize the binding of the peroxidase conjugated antibody. The results showed that the SODHAVP1/1 reacted with both the seropositive samples and neither of the seronegative samples; the SODHAV2-2 reacted with one of two seropositive samples and neither
20 seronegative sample.

In another assay, SODHAVP1/1 was used as the coating antigen in an ELISA assay with HAV seronegative and HAV seropositive human and chimpanzee sera. All four positive sera showed a greater reaction with the SODHAVP1/1 than did the four negative sera. In this assay, microtiter wells were coated with solution of 5 µg/ml of SODHAVP1/1. The coated wells were then reacted with a 1:50 dilution of the
25 various chimp and human sera for 1 hr, and were then incubated with peroxidase conjugated goat anti-human IgG (which cross-reacts with chimp IgG). The wells were finally incubated with ABTS and H₂O₂, and read on an ELISA reader at 414 nm. The results are given in absorbance units.

30	<u>Serum</u>	<u>HAV Reactive</u>	
	Human-1	-	0.211
	Human-2	-	0.346
35	Chimp-1	-	0.459
	Chimp-2	-	0.100
	Human-3	+	0.787
	Human-4	+	0.483
40	Human-5	+	0.788
	Chimp-3	+	1.224

45 D.3. Construction of Amino Acid Sequences Corresponding to Putative Epitopes

Three short amino acid sequences were synthesized based on analysis of the deduced amino acid sequence of HAV and comparison with the known epitopes of polio virus protein. The hydrophilicity and
50 structural characteristics as a function of position for both the polio polypeptide and the HAV polypeptide were compared with the aid of a computer-based analysis. "Peaks" representing putative areas of structural homology have been identified with corresponding Roman numerals and are shown in Figure 4.

Areas III and V of the polio virus polypeptide represent major antigenic sites of VP1 (Minor et al, Nature (1983) 301:674-679; Evans et al, Nature (1983) 304:459-462). Based on this information, and upon the
55 homology between HAV and polio, two polypeptides comprising sequences that give rise to the corresponding HAV peaks Va and IIIa (Figure 4) were synthesized. A third polypeptide corresponding to an HAV region with characteristics similar to those of Va and IIIa but with no homologous region in the polio sequence was also synthesized.

The three sequences constructed are as follows, where the numbers correspond to the positions designated in the deduced sequence of Figure 1.

5

Peak IIIa:

561

565

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-

10

570

575 576

His-Thr- Ser-Asp-His-Met-Ser-Cys;

15

Peak Va

591

595

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-

600

602

20

Thr-Phe-Pro-Cys;

25

No Polio Homology

606

610

615

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-

30

618

Ser-Thr-Cys.

Each of these sequences ends with a C-terminal cysteine for use in conjugation by a covalent bond to a carrier protein of sufficient size to confer immunogenicity of the peptide epitope.

The synthesized proteins were conjugated to the carrier protein, keyhole limpet hemocyanin (KLH) by disulfide linkage through the cysteine of the peptide using *m*-maleimido-benzoyl-N-hydroxysuccinimide ester (MBS) as the coupling reagent (Liu et al, *Biochem* (1979) 18:690-697; Green, N., et al, *Cell* (1982) 28:477-487). Alternatively, the peptides were coupled to KLH using glutaraldehyde (0.05-0.3%).

The conjugated peptides were injected into rabbits. Sera obtained after 31-100 days contained antibodies against the injected conjugates as assayed by ELISA. Western blots using the antisera against gels run on tissue culture-produced HAV show immunoprecipitates for the antisera produced in response to the protein corresponding to peak Va and to the protein with no polio homology. Immunoprecipitation was observed with the bands corresponding to VP1 protein.

45

D.4. Preparation of Non-Infectious (Deleted) Virions

Deleted virions can be prepared using expression vectors compatible with procaryotic, yeast, and mammalian cells.

The P1 precursor of several picornaviruses synthesized *in vitro* can be processed into individual virion proteins which then assemble into immunoreactive virion-like particles (Nicklin, et al, *Biotechnology* (1986) 4:33; Palmenberg, et al, *J. Virol* (1979) 32:770; Shih, et al, *Proc Natl Acad Sci (USA)* (1978) 75:5807; Hanecak, et al, *Proc Natl Acad Sci (USA)* (1982) 79:3973; Grubman, et al, *J Virol* (1985) 56:120).

To obtain a similar effect *in vivo*, the *E. coli* transfected with pSODHAV10-5, which produces the P1 precursor (see 1D.2) can be modified to produce the virus encoded proteinase (3C) along with the P1 protein. The resulting combination of P1 precursor coexpressed with proteinase will yield non-infectious, assembled virion-like particles.

Similar constructs expressing the P1 precursor and proteinase can be produced in yeast and

mammalian cells. For production of non-infectious virion-like particles, mammalian cells can be transfected with the HAV genome under the control of appropriate regulatory elements. By deleting the 3' end portion of the genome beyond the sequences encoding 3C (i.e., the 3D region) replicase gene is removed, thereby crippling the replication ability of the virus. The transfected cells will produce all virion proteins from the P1 and P2 region in addition to 3A, 3B and 3C. This is sufficient to generate virion-like particles, but will not generate infectious virus.

For example, an expression vector compatible with mammalian cells pHAV8161-SV7d, is constructed as shown in Figure 5. The HAV coding sequences are provided by digestion of pHAV1*, pHAV8, and pHAV16 (1D.1). The mammalian replication and control systems are provided by pSV7d (see below), which is a pBR322 derivative modified to contain the SV40 origin of replication, early promoter and polyadenylation site. It also has a polylinker with several restriction sites downstream from the SV40 promoter, as well as TAA stop codons in all three reading frames downstream from the polylinker.

In more detail, pSV7d was constructed as follows: the 400 bp BamHI/HindIII fragment containing the SV40 origin of replication and early promoter was excised from pSVgt1 (Mulligan et al, Mol Cell Biol (1981) 1:854-864) and purified. The 240 bp SV40 BclI/BamHI fragment containing SV40 polyA addition sites was excised from pSV2/dhfr (Subramani et al, J Mol Cell Biol (1981) 1:854-864) and purified. The fragments were fused through the linker:

Stop Codons

1 2 3

5' -AGCTAGATCTCCCGGGTCTAGATAAGTAAT-3'
TCTAGAGGGCCCCAGATCTATTCTACTAG

HindIII BglII SmaI XbaI BclI overhang

which contains the restriction sites shown and stop codons in all three reading frames. The resulting 670 bp. fragment, containing SV40 origin of replication, SV40 early promoter, polylinker with stop codons and SV40 polyadenylation site was closed into the BamHI site of pML, a pBR322 derivative with an about 2.5 kb deletion (Lusky and Botchan, Cell (1984) 36:391), to yield pSV6. The EcoRI and EcoRV sites in the pML sequences of pSV6 were eliminated by digestion with EcoRI and EcoRV, treatment with Bal31 nuclease to remove about 200 bp per end, and religation to yield pSV7a. (The Bal31 resection also eliminated one BamHI restriction site flanking the SV40 region approximately 200 bp away from the EcoRV site. To eliminate the other BamHI site flanking the SV40 region, pSV7a was digested with NruI, which cuts in the pML sequence upstream from the origin of replication, and recircularized by blunt end ligation to yield pSV7b.

pSV7c and pSV7d represent successive polylinker replacements. First, pSV7b was digested with StuI and XbaI, and the linker:

BglII EcoRI SmaI KpnI XbaI

5' -AGATCTCGAATTCCTCCCGGGGTACCT
TCTAGAGCTTAAGGGGCCCCCATGGAGATC

was ligated into the vector to yield pSV7c.

pSV7c was digested with BglII and XbaI, and the linker:

BglII EcoRI SmaI XbaI BamHI SalI

5' -GATCTCGAATTCCTCCCGGGTCTAGAGGATCCGTCGAC
AGCTTAAGGGGCCCCAGATCTCCTAGGCACGTGATC

was ligated to yield pSV7d.

In the construction of pHAV8161-SV7d, pHAV161 (supra) was digested with BamHI and Sall to release the 4.2 kb fragment encoding a portion of the HAV sequence. pHAV8 was digested with Sall and BamHI to provide the coding sequences upstream of the Sall site in the pHAV161 insert. These two fragments were ligated into the BamHI site of pSV7d. The resulting vector pHAV8161-SV7d places the ligated HAV sequences encoding amino acid 1-2076 under control of the SV40 promoter and directly behind the vector provided stop codon, as shown in Figure 8.

pHAV8161-SV7d may be transfected into COS cells and transient expression tested by immunofluorescence using human convalescent serum for HAV. Plasmid DNA from successful COS transformants may then be used to construct vectors for stable expression.

D.5. Hybrid Particle HAV Immunogens

U.S. Serial No. 650,323, filed 12 April 1984 and assigned to the same assignee is incorporated herein by reference. This application describes the construction of hybrid particles of hepatitis B surface antigen (HBsAg) containing inserts of foreign immunogens into a pre-surface (pre-S) coding portion in reading frame with the codons for HBsAg. Plasmid pDC101, described therein contains a portion of the pre-S/HBsAg gene, including 55 codons of the pre-S region, in a GAPDH controlled expression cassette cloned into the BamHI site of pBR322 derivative. The incorporated application describes the insertion of desired immunogens, such as the gD (glycoprotein D) antigenic site into a unique EcoRI site present in the pre-S region of pDC101 to give the hybrid plasmid pDC103. Similarly, in accordance with the present invention, desired epitopes derived from the HAV genome may be provided with suitable EcoRI linkers and inserted in proper reading frames into the EcoRI site of pDC101, or used to replace the gD codons in the pDC103 hybrid. pDC103 is deposited with ATCC and has accession no. 20726.

The construction of pDC-104, a yeast expression vector for a hybrid gene containing the sequence encoding HAV-VP1 epitope shown in Figure 6 was as follows. The HindIII/Sall fragment obtained from HAV1307 (and containing codons for amino acids 445-657 of the VP1 region) was provided with synthetic EcoRI linkers and ligated into the EcoRI site of pDC103 in place of the gD sequences. The resultant was transformed into yeast host *S. carlsbergensis* 2150-2-3 and the cultured hosts harvested. The hybrid HBV/HAV particle is detected in lysates of the yeast cells by using immobilized HBV antisera to capture the antigen and a second soluble antiserum to HAV labeled with horseradish peroxidase. Alternatively, bound anti-HAV antisera can be used to capture the particle, followed by a second labeled anti-HBV antiserum.

pWG-1, a yeast expression vector containing the coding sequence for the hybrid gene harboring the VP-1 neutralizing epitope in the particle-forming portion of the HBsAg-encoding gene is constructed similarly (see Figure 7). The DNA corresponding to the HAV epitope of the following sequence:

```

1  GATCCAGTTTTAGCAAAAGAAGGTACCTGAGACATTTCTGAATTGAAGCCTGGAGAGTCC
   GTCAAAATCGTTTCTTCATGGACTCTGTAAAGGACTTAACCTCGGACCTCTCAGG
61  AGACATACATCAGATCACATGTCTATTTATAAATTCATGGGAAGGTCTCATTTTTTGTGC
   TCTGTATGTAGTCTAGTGTACAGATAAATATTTAAGTACCCTTCCAGAGTAAAAACACG
121 ACTTTTACTTTCAATTCAATAATAAAGAGTACACATTTCCAATCG
   TGAAAATGAAAGTTAAGTTTATTATTTCTCATGTGTAAAGGTTAGCCTAG

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codes for amino acids 792-848 and is synthesized using a commercially available automated oligonucleotide synthesizer. The resulting synthetic oligomer, containing BamHI sites, is then cloned into the BglII sites of the host vector pDC101, described in U.S. 650,323 (supra), replacing a 150 bp fragment of the HBsAg coding for a dominant epitope. The resulting vector pWG-1 is transformed into *S. carlsbergensis* as above, and the transformants selected for LEU⁺. Successful transformants are cultured and grown, and the modified HBsAg particles recovered from the medium, and their bifunctional character confirmed as above.

Hybrid particle immunogens are thus prepared using fused coding sequences for HBsAg and HAV and provide enhanced immunogenicity for the HAV epitopes.

Claims

Claims for the following Contracting States : CH, DE, FR, GB, LI, NL

1. A nucleotide sequence substantially identical with that of the HAV genome as represented in Figure 1.



was ligated to yield pSV7d.

In the construction of pHAV8161-SV7d, pHAV161 (supra) was digested with BamHI and Sall to release the 4.2 kb fragment encoding a portion of the HAV sequence. pHAV8 was digested with Sall and BamHI to provide the coding sequences upstream of the Sall site in the pHAV161 insert. These two fragments were ligated into the BamHI site of pSV7d. The resulting vector pHAV8161-SV7d places the ligated HAV sequences encoding amino acid 1-2076 under control of the SV40 promoter and directly behind the vector provided stop codon, as shown in Figure 8.

pHAV8161-SV7d may be transfected into COS cells and transient expression tested by immunofluorescence using human convalescent serum for HAV. Plasmid DNA from successful COS transformants may then be used to construct vectors for stable expression.

D.5. Hybrid Particle HAV Immunogens

U.S. Serial No. 650,323, filed 12 April 1984 and assigned to the same assignee is incorporated herein by reference. This application describes the construction of hybrid particles of hepatitis B surface antigen (HBsAg) containing inserts of foreign immunogens into a pre-surface (pre-S) coding portion in reading frame with the codons for HBsAg. Plasmid pDC101, described therein contains a portion of the pre-S/HBsAg gene, including 55 codons of the pre-S region, in a GAPDH controlled expression cassette cloned into the BamHI site of pBR322 derivative. The incorporated application describes the insertion of desired immunogens, such as the gD (glycoprotein D) antigenic site into a unique EcoRI site present in the pre-S region of pDC101 to give the hybrid plasmid pDC103. Similarly, in accordance with the present invention, desired epitopes derived from the HAV genome may be provided with suitable EcoRI linkers and inserted in proper reading frames into the EcoRI site of pDC101, or used to replace the gD codons in the pDC103 hybrid. pDC103 is deposited with ATCC and has accession no. 20726.

The construction of pDC-104, a yeast expression vector for a hybrid gene containing the sequence encoding HAV-VP1 epitope shown in Figure 6 was as follows. The HindIII/Sall fragment obtained from HAV1307 (and containing codons for amino acids 445-657 of the VP1 region) was provided with synthetic EcoRI linkers and ligated into the EcoRI site of pDC103 in place of the gD sequences. The resultant was transformed into yeast host *S. carlsbergensis* 2150-2-3 and the cultured hosts harvested. The hybrid HBV/HAV particle is detected in lysates of the yeast cells by using immobilized HBV antisera to capture the antigen and a second soluble antiserum to HAV labeled with horseradish peroxidase. Alternatively, bound anti-HAV antisera can be used to capture the particle, followed by a second labeled anti-HBV antiserum.

pWG-1, a yeast expression vector containing the coding sequence for the hybrid gene harboring the VP-1 neutralizing epitope in the particle-forming portion of the HBsAg-encoding gene is constructed similarly (see Figure 7). The DNA corresponding to the HAV epitope of the following sequence:

```

1  GATCCAGTTTTASCAAAGAAGGTACCTGAGACATTTCTGAATTGAAGCCTGGAGAGTCC
   GTCAAAATCGTTTCTTCCATGGACTCTGTAAAGGACTTAACTTCGGACCTCTCAGG
61  AGACATACATCAGATCACATGTCTATTTATAAAATTCATGGGAAGGTCTCATTTTTTGTGC
   TCTGTATGTAGTCTAGTGTACAGATAAATATTTAAGTACCCCTTCCAGAGTAAAAACACG
40 121  ACTTTTACTTTCAATTCAAATAATAAAGAGTACACATTTCCAATCG
   TGAAAATGAAAGTTAAGTTTATTATTTCTCATGTGTAAAGGTTAGCCTAG

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codes for amino acids 792-848 and is synthesized using a commercially available automated oligonucleotide synthesizer. The resulting synthetic oligomer, containing BamHI sites, is then cloned into the BglII sites of the host vector pDC101, described in U.S. 650,323 (supra), replacing a 150 bp fragment of the HBsAg coding for a dominant epitope. The resulting vector pWG-1 is transformed into *S. carlsbergensis* as above, and the transformants selected for LEU⁺. Successful transformants are cultured and grown, and the modified HBsAg particles recovered from the medium, and their bifunctional character confirmed as above.

Hybrid particle immunogens are thus prepared using fused coding sequences for HBsAg and HAV and provide enhanced immunogenicity for the HAV epitopes.

Claims

Claims for the following Contracting States : CH, DE, FR, GB, LI, NL

1. A nucleotide sequence substantially identical with that of the HAV genome as represented in Figure 1.



2. A nucleotide sequence encoding a viral polypeptide substantially identical with that encoded by the HAV genomic sequence shown in Figure 1.
3. A recombinant expression system comprising a coding portion derived from the sequence of Claim 1 operably linked to a control sequence compatible with a desired host; the coding portion being any portion other than fragments substantially identical to nucleotides 328-3305, 2074-3047, 1812-3305, 1969-3047, 1806-3305, 1808-1834, 2207-2239, 2288-2323, 2324-2413, 2474-2530 2675-2716 or 23-3295 of the sequence represented in Figure 1.
4. Recombinant host cells transformed with the expression system of Claim 3.
5. Protein produced by the cells of Claim 4.
6. The system of Claim 3 which further includes, contiguous to said coding portion, and in reading frame therewith, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.
7. A recombinant expression system comprising a coding portion derived from the sequence of Claim 1 operably linked to a control sequence compatible with a desired host which further includes, upstream of said coding portion, and separated by a stop codon, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.
8. The system of claim 6 or 7 wherein the fused DNA sequence encodes human superoxide dismutase or portion thereof.
9. The system of claims 6 or 7 wherein the fused DNA sequence encodes a non-HAV particle forming protein.
10. Recombinant host cells transformed with the system of Claim 6 or 7.
11. Protein produced by the cells of Claim 10.
12. The system of Claim 3 wherein the coding portion is the nucleotide sequence of Claim 1 between nucleotide positions selected from the group consisting of 2072-3201; 1182-3201; and 734-3201.
13. An antigenic peptide comprising a sequence derived from the sequence of claim 1 covalently linked at the C terminus to cysteine which is selected from the group:

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-
Ser-Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-
Pro-Cys; and

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-
Ser-Thr-Cys.

14. The protein according to claim 5, wherein said protein contains an epitope of HAV coupled to a non HAV particle forming protein to form a complex, such that the complex is capable of forming a particle immunogenic against HAV infection.
15. The protein according to Claim 14 wherein the epitope is derived from amino acids 445-657 or 792-848 of the HAV polypeptide sequence.



16. An oligonucleotide useful for detecting the presence of HAV in a biological sample which comprises a DNA sequence derived from the nucleotide sequence in Figure 1; the oligonucleotide being a fragment according to claim 3.
17. A protein according to any one of Claims 5, 11 and 13-15 for use in a vaccine effective against Hepatitis A virus.
18. An expression system for a deleted HAV virion which comprises a nucleotide sequence encoding the P1 precursor protein as shown in Figure 1, said nucleotide sequence operably linked to suitable control sequences, and transformed into a host cell capable of producing a protease effective in cleaving said P1 precursor to obtain a deleted HAV virion.
19. The expression system of claim 18 wherein said protease is encoded by the 3C HAV region of Figure 1.
20. An expression system according to claim 18 wherein said deleted virion is encoded by a DNA corresponding to nucleotides 734-5767 of the sequence represented in Figure 1.
21. Protein produced by an expression system according to any one of claims 18 to 20.
22. An expression system according to claim 3 wherein said nucleotide sequence encodes VP2.
23. A process for obtaining protein comprising HAV sequences which comprises culturing cells according to claim 4 or claim 10, or cells containing an expression system according to claim 18, and recovering the protein thus obtained.

Claims for the following Contracting States : AT, BE, LU, SE

1. A nucleotide sequence substantially identical with that of the HAV genome as represented in Figure 1.
2. A nucleotide sequence encoding a viral polypeptide substantially identical with that encoded by the HAV genomic sequence shown in Figure 1.
3. A recombinant expression system comprising a coding portion derived from the sequence of Claim 1 operably linked to a control sequence compatible with a desired host; the coding portion being any portion other than fragments substantially identical to nucleotides 23-3295 of the sequence represented in Figure 1.
4. Recombinant host cells transformed with the expression system of Claim 3.
5. Protein produced by the cells of Claim 4.
6. The system of Claim 3 which further includes, contiguous to said coding portion, and in reading frame therewith, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.
7. The system of Claim 3 which further includes, upstream of said coding portion, and separated by a stop codon, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.
8. The system of Claim 6 or 7 wherein the fused DNA sequence encodes human superoxide dismutase or portion thereof.
9. The system of claims 6 or 7 wherein the fused DNA sequence encodes a non-HAV particle forming protein.
10. Recombinant host cells transformed with the system of Claim 6 or 7.
11. Protein produced by the cells of Claim 10.



12. The system of Claim 3 wherein the coding portion is the nucleotide sequence of Claim 1 between nucleotide positions selected from the group consisting of 2072-3201; 1182-3201; and 734-3201.

13. An antigenic peptide comprising a sequence derived from the sequence of claim 1 covalently linked at the C terminus to cysteine which is selected from the group:

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-
Ser-Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-
Pro-Cys; and

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-
Ser-Thr-Cys.

14. The protein according to claim 5, wherein said protein contains an epitope of HAV coupled to a non HAV particle forming protein to form a complex, such that the complex is capable of forming a particle immunogenic against HAV infection.

15. The protein according to Claim 14 wherein the epitope is derived from amino acids 445-657 or 792-848 of the HAV polypeptide sequence.

16. An oligonucleotide useful for detecting the presence of HAV in a biological sample which comprises a DNA sequence derived from the nucleotide sequence in Figure 1; the oligonucleotide being other than the fragment substantially identical to nucleotides 23-3295 of the sequence represented in Figure 1.

17. A protein according to any one of Claims 5, 11 and 13-15 for use in a vaccine effective against Hepatitis A virus.

18. An expression system for a deleted HAV virion which comprises a nucleotide sequence encoding the P1 precursor protein as shown in Figure 1, said nucleotide sequence operably linked to suitable control sequences, and transformed into a host cell capable of producing a protease effective in cleaving said P1 precursor to obtain a deleted HAV virion.

19. The expression system of claim 18 wherein said protease is encoded by the 3C HAV region of Figure 1.

20. An expression system according to claim 18 wherein said deleted virion is encoded by a DNA corresponding to nucleotides 734-5767 of the sequence represented in Figure 1.

21. Protein produced by an expression system according to any one of claims 18 to 20.

22. An expression system which comprises a nucleotide sequence encoding a protein selected from the group consisting of VP1, VP2 and VP3 operably linked to suitable control sequences.

23. A process for obtaining protein comprising HAV sequences which comprises culturing cells according to claim 4 or claim 10, or cells containing an expression system according to claim 18, and recovering the protein thus obtained.

Claims for the following Contracting State : IT

1. A nucleotide sequence substantially identical with that of the HAV genome as represented in Figure 1.



2. A nucleotide sequence encoding a viral polypeptide substantially identical with that encoded by the HAV genomic sequence shown in Figure 1.
3. A recombinant expression system comprising a coding portion derived from the sequence of Claim 1 operably linked to a control sequence compatible with a desired host; the coding portion being any portion other than fragments substantially identical to nucleotides 328-3305, 2074-3047, 1812-3305, 1969-3047, 1806-3305, 1808-1834, 2207-2239, 2288-2323, 2324-2413, 2474-2530 or 2675-2716 of the sequence represented in Figure 1.
4. Recombinant host cells transformed with the expression system of Claim 3.
5. Protein produced by the cells of Claim 4.
6. The system of Claim 3 which further includes, contiguous to said coding portion, and in reading frame therewith, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.
7. A recombinant expression system comprising a coding portion derived from the sequence of Claim 1 operably linked to a control sequence compatible with a desired host which further includes, upstream of said coding portion, and separated by a stop codon, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.
8. The system of claim 6 or 7 wherein the fused DNA sequence encodes human superoxide dismutase or portion thereof.
9. The system of claims 6 or 7 wherein the fused DNA sequence encodes a non-HAV particle forming protein.
10. Recombinant host cells transformed with the system of Claim 6 or 7.
11. Protein produced by the cells of Claim 10.
12. The system of Claim 3 wherein the coding portion is the nucleotide sequence of Claim 1 between nucleotide positions selected from the group consisting of 2072-3201; 1182-3201; and 734-3201.
13. An antigenic peptide comprising a sequence derived from the sequence of claim 1 covalently linked at the C terminus to cysteine which is selected from the group:

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-
Ser-Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-
Pro-Cys; and

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-
Ser-Thr-Cys.

14. The protein according to claim 5, wherein said protein contains an epitope of HAV coupled to a non HAV particle forming protein to form a complex, such that the complex is capable of forming a particle immunogenic against HAV infection.
15. The protein according to Claim 14 wherein the epitope is derived from amino acids 445-657 or 792-848 of the HAV polypeptide sequence.



16. An oligonucleotide useful for detecting the presence of HAV in a biological sample which comprises a DNA sequence derived from the nucleotide sequence in Figure 1; the oligonucleotide being a fragment according to claim 3.
17. A protein according to any one of Claims 5, 11 and 13-15 for use in a vaccine effective against Hepatitis A virus.
18. An expression system for a deleted HAV virion which comprises a nucleotide sequence encoding the P1 precursor protein as shown in Figure 1, said nucleotide sequence operably linked to suitable control sequences, and transformed into a host cell capable of producing a protease effective in cleaving said P1 precursor to obtain a deleted HAV virion.
19. The expression system of claim 18 wherein said protease is encoded by the 3C HAV region of Figure 1.
20. An expression system according to claim 18 wherein said deleted virion is encoded by a DNA corresponding to nucleotides 734-5767 of the sequence represented in Figure 1.
21. Protein produced by an expression system according to any one of claims 18 to 20.
22. An expression system according to claim 3 wherein said nucleotide sequence encodes VP1, VP2 or VP3.
23. A process for obtaining protein comprising HAV sequences which comprises culturing cells according to claim 4 or claim 10, or cells containing an expression system according to claim 18, and recovering the protein thus obtained.

Revendications

Revendications pour les Etats contractants suivants : CH, DE, FR, GB, LI, NL

1. Séquence nucléotidique sensiblement identique à celle du génome HAV telle que représentée à la figure 1.
2. Séquence nucléotidique codant pour un polypeptide viral sensiblement identique à celui qui est codé par la séquence génomique HAV représentée à la figure 1.
3. Système d'expression recombinant comprenant une partie codante dérivée de la séquence selon la revendication 1 liée de façon opératoire à une séquence de contrôle compatible avec un hôte désiré; la partie codante étant n'importe quelle partie autre que les fragments sensiblement identiques aux nucléotides 328-3305, 2074-3047, 1812-3305, 1969-3047, 1806-3305, 1808-1834, 2207-2239, 2288-2323, 2324-2413, 2474-2530, 2675-2716 ou 23-3295 de la séquence représentée à la figure 1.
4. Cellules hôtes recombinantes transformées par le système d'expression selon la revendication 3.
5. Protéine produite par les cellules selon la revendication 4.
6. Système selon la revendication 3 qui comprend en outre, contiguë à ladite partie codante, et dans un cadre de lecture avec elle, une séquence nucléotidique fusionnée codant pour une protéine non codée par HAV ou une de ses parties.
7. Système d'expression recombinant comprenant une partie codante dérivée de la séquence selon la revendication 1 liée de façon opératoire à une séquence de contrôle compatible avec un hôte désiré qui inclut en outre, en amont de ladite partie codante, et séparée par un codon d'arrêt, une séquence nucléotidique fusionnée codant pour une protéine non codée par HAV ou une de ses parties.
8. Système selon la revendication 6 ou 7 dans lequel la séquence d'ADN fusionnée code pour la superoxyde dismutase humaine ou une de ses parties.



9. Système selon les revendications 6 ou 7 dans lequel la séquence d'ADN fusionnée code pour une protéine non formatrice de particules HAV.
10. Cellules hôtes recombinantes transformées par le système selon la revendication 6 ou 7.
11. Protéine produite par les cellules selon la revendication 10.
12. Système selon la revendication 3 dans lequel la partie codante est la séquence nucléotidique selon la revendication 1 située entre les positions nucléotidiques choisies dans le groupe constitué par 2072-3201; 1182-3201; et 734-3201.
13. Peptide antigénique comprenant une séquence dérivée de la séquence selon la revendication 1 liée de façon covalente par le groupement C-terminal à une cystéine, choisie dans le groupe:
- Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-Ser-Asp-His-Met-Ser-Cys;
- Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-Pro-Cys; et
- Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-Ser-Thr-Cys.
14. Protéine selon la revendication 5, dans laquelle ladite protéine contient un épitope de HAV couplé à une protéine non formatrice de particules HAV pour former un complexe, de manière que le complexe soit capable de former une particule immunogène contre l'infection par HAV.
15. Protéine selon la revendication 14 dans laquelle l'épitope est dérivé des acides aminés 445-657 ou 792-848 de la séquence polypeptidique de HAV.
16. Oligonucléotide utile pour détecter la présence de HAV dans un échantillon biologique qui comprend une séquence d'ADN dérivée de la séquence nucléotidique à la figure 1; l'oligonucléotide étant un fragment selon la revendication 3.
17. Protéine selon l'une quelconque des revendications 5, 11 et 13-15 destinée à l'utilisation dans un vaccin efficace contre le virus de l'hépatite A.
18. Système d'expression pour un virion de HAV délété qui comprend une séquence nucléotidique codant pour la protéine précurseur P1 telle que représentée à la figure 1, ladite séquence nucléotidique étant liée de façon opératoire à des séquences de contrôle appropriées, et transformée dans une cellule hôte capable de produire une protéase efficace pour cliver ledit précurseur P1 afin d'obtenir un virion de HAV délété.
19. Système d'expression selon la revendication 18 dans lequel ladite protéase est codée par la région 3C de HAV à la figure 1.
20. Système d'expression selon la revendication 18 dans lequel ledit virion délété est codé par un ADN correspondant aux nucléotides 734-5767 de la séquence représentée à la figure 1.
21. Protéine produite par un système d'expression selon l'une quelconque des revendications 18 à 20.
22. Système d'expression selon la revendication 3 dans lequel ladite séquence nucléotidique code pour la



VP2.

23. Procédé d'obtention d'une protéine comprenant des séquences de HAV dans lequel on cultive des cellules selon la revendication 4 ou la revendication 10, ou des cellules contenant un système d'expression selon la revendication 18, et on recueille la protéine ainsi obtenue.

Revendications pour les Etats contractants suivants : AT, BE, LU, SE

1. Séquence nucléotidique sensiblement identique à celle du génome HAV telle que représentée à la figure 1.
2. Séquence nucléotidique codant pour un polypeptide viral sensiblement identique à celui qui est codé par la séquence génomique HAV représentée à la figure 1.
3. Système d'expression recombinant comprenant une partie codante dérivée de la séquence selon la revendication 1 liée de façon opératoire à une séquence de contrôle compatible avec un hôte désiré; la partie codante étant n'importe quelle partie autre que les fragments sensiblement identiques aux nucléotides 23-3295 de la séquence représentée à la figure 1.
4. Cellules hôtes recombinantes transformées par le système d'expression selon la revendication 3.
5. Protéine produite par les cellules selon la revendication 4.
6. Système selon la revendication 3 qui comprend en outre, contiguë à ladite partie codante, et dans un cadre de lecture avec elle, une séquence nucléotidique fusionnée codant pour une protéine non codée par HAV ou une de ses parties.
7. Système selon la revendication 3 qui comprend en outre, en amont de ladite partie codante, et séparée par un codon d'arrêt, une séquence nucléotidique fusionnée codant pour une protéine non codée par HAV ou une de ses parties.
8. Système de la revendication 6 ou 7 dans lequel la séquence d'ADN fusionnée code pour la superoxyde dismutase humaine ou une de ses parties.
9. Système selon les revendications 6 ou 7 dans lequel la séquence d'ADN fusionnée code pour une protéine non formatrice de particules HAV.
10. Cellules hôtes recombinantes transformées par le système selon les revendications 6 ou 7.
11. Protéine produite par les cellules selon la revendication 10.
12. Système selon la revendication 3 dans lequel la partie codante est la séquence nucléotidique selon la revendication 1 située entre les positions nucléotidiques choisies dans le groupe constitué par 2072-3201; 1182-3201; et 734-3201.
13. Peptide antigénique comprenant une séquence, dérivée de la séquence selon la revendication 1, liée de façon covalente par le groupement C-terminal à une cystéine, choisie dans le groupe:



Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-

Ser-Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-

Pro-Cys; et

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-

Ser-Thr-Cys.

14. Protéine selon la revendication 5, dans laquelle ladite protéine contient un épitope de HAV couplé à une protéine non formatrice de particules HAV pour former un complexe, de manière que le complexe soit capable de former une particule immunogène contre l'infection par HAV.
15. Protéine selon la revendication 14 dans laquelle l'épitope est dérivé des acides aminés 445-657 ou 792-848 de la séquence polypeptidique de HAV.
16. Oligonucléotide utile pour détecter la présence de HAV dans un échantillon biologique qui comprend une séquence d'ADN dérivée de la séquence nucléotidique à la figure 1; l'oligonucléotide étant autre que le fragment sensiblement identique aux nucléotides 23-3295 de la séquence représentée à la figure 1.
17. Protéine selon l'une quelconque des revendications 5, 11 et 13-15 destinée à l'utilisation dans un vaccin efficace contre le virus de l'hépatite A.
18. Système d'expression d'un virion de HAV délété qui comprend une séquence nucléotidique codant pour la protéine précurseur P1 telle que représentée à la figure 1, ladite séquence nucléotidique étant liée de façon opératoire à des séquences de contrôle appropriées, et transformée dans une cellule hôte capable de produire une protéase efficace pour cliver ledit précurseur P1 afin d'obtenir un virion de HAV délété.
19. Système d'expression de la revendication 18 dans lequel ladite protéase est codée par la région 3C de HAV à la figure 1.
20. Système d'expression selon la revendication 18 dans lequel ledit virion délété est codé par un ADN correspondant aux nucléotides 734-5767 de la séquence représentée à la figure 1.
21. Protéine produite par un système d'expression selon l'une quelconque des revendications 18 à 20.
22. Système d'expression qui comprend une séquence nucléotidique codant pour une protéine choisie dans le groupe constitué par VP1, VP2 et VP3 liée de façon opératoire à des séquences de contrôle appropriées.
23. Procédé d'obtention d'une protéine comprenant des séquences de HAV dans lequel on cultive des cellules selon la revendication 4 ou la revendication 10, ou des cellules contenant un système d'expression selon la revendication 18, et on recueille la protéine ainsi obtenue.

Revendications pour l'Etat contractant suivant : IT

1. Séquence nucléotidique sensiblement identique à celle du génome HAV telle que représentée à la figure 1.
2. Séquence nucléotidique codant pour un polypeptide viral sensiblement identique à celui codé par la



séquence génomique HAV représentée à la figure 1.

3. Système d'expression recombinant comprenant une partie codante dérivée de la séquence selon la revendication 1 liée de façon opératoire à une séquence de contrôle compatible avec un hôte désiré; la partie codante étant n'importe quelle partie autre que les fragments sensiblement identiques aux nucléotides 328-3305, 2074-3047, 1812-3305, 1969-3047, 1806-3305, 1808-1834, 2207-2239, 2288-2323, 2324-2413, 2474-2530 ou 2675-2716 de la séquence représentée à la figure 1.
4. Cellules hôtes recombinantes transformées par le système d'expression selon la revendication 3.
5. Protéine produite par les cellules selon la revendication 4.
6. Système selon la revendication 3 qui comprend en outre, contiguë à ladite partie codante, et dans un cadre de lecture avec elle, une séquence nucléotidique fusionnée codant pour une protéine non codée par HAV ou une de ses parties.
7. Système d'expression recombinant comprenant une partie codante dérivée de la séquence selon la revendication 1 liée de façon opératoire à une séquence de contrôle compatible avec un hôte désiré qui inclut en outre, en amont de ladite partie codante, et séparée par un codon d'arrêt, une séquence nucléotidique fusionnée codant pour une protéine non codée par HAV ou une de ses parties.
8. Système selon la revendication 6 ou 7 dans lequel la séquence d'ADN fusionnée code pour la superoxyde dismutase humaine ou une de ses parties.
9. Système selon les revendications 6 ou 7 dans lequel la séquence d'ADN fusionnée code pour une protéine non formatrice de particules HAV.
10. Cellules hôtes recombinantes transformées par le système selon la revendication 6 ou 7.
11. Protéine produite par les cellules selon la revendication 10.
12. Système selon la revendication 3 dans lequel la partie codante est la séquence nucléotidique selon la revendication 1 située entre les positions nucléotidiques choisies dans le groupe constitué par 2072-3201; 1182-3201; et 734-3201.
13. Peptide antigénique comprenant une séquence, dérivée de la séquence selon la revendication 1, liée de façon covalente par le groupement C-terminal à une cystéine, choisie dans le groupe:

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-Ser-Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-Pro-Cys; et

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-Ser-Thr-Cys.

14. Protéine selon la revendication 5, dans laquelle ladite protéine contient un épitope de HAV couplé à une protéine non formatrice de particules HAV pour former un complexe, de manière que le complexe soit capable de former une particule immunogène contre l'infection par HAV.
15. Protéine selon la revendication 14 dans laquelle l'épitope est dérivé des acides aminés 445-657 ou



792-848 de la séquence polypeptidique de HAV.

16. Oligonucléotide utile pour détecter la présence de HAV dans un échantillon biologique qui comprend une séquence d'ADN dérivée de la séquence nucléotidique à la figure 1; l'oligonucléotide étant un fragment selon la revendication 3.
17. Protéine selon l'une quelconque des revendications 5, 11 et 13-15 destinée à l'utilisation dans un vaccin efficace contre le virus de l'hépatite A.
18. Système d'expression pour un virion de HAV délété qui comprend une séquence nucléotidique codant pour la protéine précurseur P1 telle que représentée à la figure 1, ladite séquence nucléotidique étant liée de façon opératoire à des séquences de contrôle appropriées, et transformée dans une cellule hôte capable de produire une protéase efficace pour cliver ledit précurseur P1 afin d'obtenir un virion de HAV délété.
19. Système d'expression selon la revendication 18 dans lequel ladite protéase est codée par la région 3C de HAV à la figure 1.
20. Système d'expression selon la revendication 18 dans lequel ledit virion délété est codé par un ADN correspondant aux nucléotides 734-5767 de la séquence représentée à la figure 1.
21. Protéine produite par un système d'expression selon l'une quelconque des revendications 18 à 20.
22. Système d'expression selon la revendication 3 dans lequel ladite séquence nucléotidique code pour la VP1, la VP2 ou la VP3.
23. Procédé d'obtention d'une protéine comprenant des séquences de HAV dans lequel on cultive des cellules selon la revendication 4 ou la revendication 10, ou des cellules contenant un système d'expression selon la revendication 18, et on recueille la protéine ainsi obtenue.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : CH, DE, FR, GB, LI, NL

1. Nukleotidsequenz, im wesentlichen identisch mit der des HAV-Genoms, wie in Figur 1 dargestellt.
2. Nukleotidsequenz, codierend für ein virales Polypeptid, das im wesentlichen mit dem identisch ist, das von der HAV-Genomsequenz, wie in Figur 1 gezeigt, codiert wird.
3. Rekombinantes Expressionssystem, umfassend einen codierenden Teil, abgeleitet von der Sequenz nach Anspruch 1, in ausführbarer Weise geknüpft an eine mit dem gewünschten Wirt kompatible Kontrollsequenz, wobei der codierende Teil jeder beliebige Teil außer Fragmente, die im wesentlichen identisch mit den Nukleotiden 328-3305, 2074-3047, 1812-3305, 1969-3047, 1806-3305, 1808-1834, 2207-2239, 2288-2323, 2324-2413, 2474-2530, 2675-2716 oder 23-3295 der Sequenz nach Figur 1 sind, ist.
4. Rekombinante Wirtszellen, transformiert mit dem Expressionssystem nach Anspruch 3.
5. Protein, gebildet von den Zellen nach Anspruch 4.
6. System nach Anspruch 3, dadurch gekennzeichnet, daß es weiterhin, angrenzend an den codierenden Teil und im Leseraster damit eine fusionierte Nukleotidsequenz, die für ein nicht-HAV-codiertes Protein oder einen Teil davon codiert, enthält.
7. Rekombinantes Expressionssystem, umfassend einen codierenden Teil, abgeleitet von der Sequenz nach Anspruch 1, in ausführbarer Weise gebunden an eine mit dem gewünschten Wirt verträgliche Kontrollsequenz, enthaltend weiterhin stromaufwärts des codierenden Teils und getrennt durch ein Stoppcodon, eine fusionierte Nukleotidsequenz, die für ein nicht-HAV-codiertes Protein oder einen Teil davon codiert.



8. System nach Anspruch 6 oder 7, dadurch **gekennzeichnet**, daß die fusionierte DNA-Sequenz für menschliche Superoxid-Dismutase oder einen Teil davon codiert.
9. System nach den Ansprüchen 6 oder 7, dadurch **gekennzeichnet**, daß die fusionierte DNA-Sequenz für ein nicht-HAV-Teilchen-bildendes Protein codiert.
10. Rekombinante Wirtszellen, transformiert mit dem System nach Anspruch 6 oder 7.
11. Protein, gebildet von den Zellen nach Anspruch 10.
12. System nach Anspruch 3, dadurch **gekennzeichnet**, daß der codierende Teil die Nukleotidsequenz nach Anspruch 1 zwischen den Nukleotidpositionen, ausgewählt aus der Gruppe bestehend aus 2072-3201, 1182-3201 und 734-3201, ist.
13. Antigenes Peptid, umfassend eine Sequenz, abgeleitet von der Sequenz nach Anspruch 1, in covalenter Bindung an den C-Terminus an Cystein, ausgewählt aus der Gruppe:

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-Ser-
 Asp-His-Met-Ser-Cys;
 Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-Pro-
 Cys; und
 Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-Ser-

Thr-Cys.

14. Protein nach Anspruch 5, dadurch **gekennzeichnet**, daß das Protein ein Epitop von HAV in Kopplung an ein nicht-HAV-Teilchen-bildendes Protein, unter Bildung eines Komplexes enthält, so daß der Komplex in der Lage ist, ein Teilchen zu bilden, das gegen eine HAV-Infektion immunogen ist.
15. Protein nach Anspruch 14, dadurch **gekennzeichnet**, daß das Epitop von den Aminosäuren 445-657 oder 792-848 der HAV-Polypeptidsequenz abgeleitet ist.
16. Oligonukleotid, geeignet zur Detektion auf die Anwesenheit von HAV in einer biologischen Probe, umfassend eine DNA-Sequenz, abgeleitet von der Nukleotidsequenz nach Figur 1, wobei das Oligonukleotid ein Fragment nach Anspruch 3 ist.
17. Protein nach einem der Ansprüche 5, 11 und 13 bis 15 zur Verwendung in einem Impfstoff, der gegen das Hepatitis-A-Virus wirksam ist.
18. Expressionssystem für ein deletiertes HAV-Virion, umfassend eine Nukleotidsequenz, codierend für das P1-Precursor-Protein, wie in Figur 1 gezeigt, wobei die Nukleotidsequenz in ausführbarer Weise an geeignete Kontrollsequenzen gebunden ist und in eine Wirtszelle transformiert ist, die in der Lage ist, eine Protease zu erzeugen, die wirksam den P1-Precursor unter Erhalt eines deletierten HAV-Virions spaltet.
19. Expressionssystem nach Anspruch 18, dadurch **gekennzeichnet**, daß die Protease von der 3C HAV-Region der Figur 1 codiert ist.
20. Expressionssystem nach Anspruch 18, dadurch **gekennzeichnet**, daß das deletierte Virion von einer DNA, entsprechend den Nukleotiden 734-5767 der Sequenz nach Figur 1 codiert ist.



21. Protein, gebildet von einem Expressionssystem nach einem der Ansprüche 18 bis 20.
 22. Expressionssystem nach Anspruch 3, dadurch gekennzeichnet, daß die Nukleotidsequenz für VP2 codiert.
 23. Verfahren zur Herstellung eines Proteins, umfassend HAV-Sequenzen, dadurch gekennzeichnet, daß man Zellen nach Anspruch 4 oder Anspruch 10 oder Zellen, die ein Expressionssystem nach Anspruch 18 enthalten, kultiviert und das so erhaltene Protein gewinnt.
- 10 **Patentansprüche für folgende Vertragsstaaten : AT, BE, LU, SE**
1. Nukleotidsequenz, im wesentlichen identisch mit der des HAV-Genoms, wie in Figur 1 dargestellt.
 2. Nukleotidsequenz, codierend für ein virales Polypeptid, das im wesentlichen mit dem identisch ist, das von der HAV-Genomsequenz, wie in Figur 1 gezeigt, codiert wird.
 3. Rekombinantes Expressionssystem, umfassend einen codierenden Teil, abgeleitet von der Sequenz nach Anspruch 1, in ausführbarer Weise geknüpft an eine mit dem gewünschten Wirt kompatible Kontrollsequenz, wobei der codierende Teil ein anderer als die Fragmente, die im wesentlichen den Nukleotiden 23-3295 der Sequenz nach Figur 1 identisch sind, ist.
 4. Rekombinante Wirtszellen, transformiert mit dem Expressionssystem nach Anspruch 3.
 5. Protein, gebildet von den Zellen nach Anspruch 4.
 6. System nach Anspruch 3, dadurch gekennzeichnet, daß es weiterhin, angrenzend an den codierenden Teil und im Leseraster damit eine fusionierte Nukleotidsequenz, die für ein nicht-HAV-codiertes Protein oder einen Teil davon codiert, enthält.
 7. System nach Anspruch 3, dadurch gekennzeichnet, daß es weiterhin stromaufwärts des codierenden Teils und abgetrennt durch ein Stopcodon eine fusionierte Nukleotidsequenz, die für ein nicht-HAV-codiertes Protein oder einen Teil davon codiert, umfaßt.
 8. System nach Anspruch 6 oder 7, dadurch gekennzeichnet, daß die fusionierte DNA-Sequenz für Human-Superoxid-Dismutase oder einen Teil davon codiert.
 9. System nach Anspruch 6 oder 7, dadurch gekennzeichnet, daß die fusionierte DNA-Sequenz für ein nicht-HAV-Teilchen-bildendes Protein codiert.
 10. Rekombinante Wirtszellen, transformiert mit dem System nach Anspruch 6 oder 7.
 11. Protein, gebildet von den Zellen nach Anspruch 10.
 12. System nach Anspruch 3, dadurch gekennzeichnet, daß der codierende Teil die Nukleotidsequenz nach Anspruch 1 zwischen die Nukleotidpositionen, ausgewählt aus der Gruppe bestehend aus 2072-3201, 1182-3201 und 734-3201, ist.
 13. Antigenes Peptid, umfassend eine Sequenz, abgeleitet von der Sequenz nach Anspruch 1 in covalenter Bindung an den C-Terminus an Cystein, ausgewählt aus der Gruppe



Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-Ser-
Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-Pro-
Cys; und

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-Ser-
Thr-Cys.

14. Protein nach Anspruch 5, dadurch **gekennzeichnet**, daß das Protein ein Epitop von HAV in Kopplung an ein nicht-HAV-Teilchen-bildendes Protein, unter Bildung eines Komplexes enthält, so daß der Komplex in der Lage ist, ein Teilchen zu bilden, das gegen eine HAV-Infektion immunogen ist.

15. Protein nach Anspruch 14, dadurch **gekennzeichnet**, daß das Epitop von den Aminosäuren 445-657 oder 792-848 der HAV-Polypeptidsequenz abgeleitet ist.

16. Oligonukleotid, geeignet zur Detektion auf die Anwesenheit von HAV in einer biologischen Probe, umfassend eine DNA-Sequenz, abgeleitet von der Nukleotidsequenz in Figur 1, wobei das Oligonukleotid ein anderes ist, als das Fragment das im wesentlichen mit den Nukleotiden 23-3295 der Sequenz nach Figur 1 identisch ist.

17. Protein nach einem der Ansprüche 5, 11 und 13 bis 15 zur Verwendung in einem Impfstoff, der wirksam gegen das Hepatitis-A-Virus ist.

18. Expressionssystem für ein deletiertes HAV-Virion, umfassend eine Nukleotidsequenz die für das P1-Precursor-Protein, wie in Figur 1 gezeigt, codiert, wobei die Nukleotidsequenz in ausführbarer Weise an geeignete Kontrollsequenzen gebunden ist und in eine Wirtszelle transformiert ist, die in der Lage ist, eine Protease die den P1-Precursor unter Erhalt eines deletierten HAV-Virions effektiv zu spalten, zu bilden.

19. Expressionssystem nach Anspruch 18, dadurch **gekennzeichnet**, daß die Protease von der 3C HAV-Region nach Figur 1 codiert ist.

20. Expressionssystem nach Anspruch 18, dadurch **gekennzeichnet**, daß das deletierte Virion von einer DNA, entsprechend den Nukleotiden 734-5767 der Sequenz nach Figur 1 codiert ist.

21. Protein, gebildet von einem Expressionssystem nach einem der Ansprüche 18 bis 20.

22. Expressionssystem, umfassend eine Nukleotidsequenz, codierend für ein Protein, ausgewählt aus der Gruppe, bestehend aus VP1, VP2 und VP3 in ausführbarer Weise gebunden an geeignete Kontrollsequenzen.

23. Verfahren zur Herstellung eines Proteins, umfassend HAV-Sequenzen, dadurch **gekennzeichnet**, daß man Zellen nach Anspruch 4 oder 10 oder Zellen, die ein Expressionssystem nach Anspruch 18 enthalten, kultiviert und das so erhaltene Protein gewinnt.

Patentansprüche für folgenden Vertragsstaat : IT

1. Nukleotidsequenz, im wesentlichen identisch mit der des HAV-Genoms, wie in Figur 1 dargestellt.

2. Nukleotidsequenz, codierend für ein virales Polypeptid, das im wesentlichen mit dem identisch ist, das von der HAV-Genomsequenz, wie in Figur 1 gezeigt, codiert wird.

3. Rekombinantes Expressionssystem, umfassend einen codierenden Teil, abgeleitet von der Sequenz nach Anspruch 1, in ausführbarer Weise geknüpft an eine mit dem gewünschten Wirt kompatible



Kontrollsequenz, wobei der codierende Teil jeder beliebige Teil außer Fragmente, die im wesentlichen identisch mit den Nukleoliden 328-3305, 2074-3047, 1812-3305, 1969-3047, 1806-3305, 1808-1834, 2207-2239, 2288-2323, 2324-2413, 2474-2530 oder 2675-2716 der Sequenz nach Figur 1 sind, ist.

- 5 4. Rekombinante Wirtszellen, transformiert mit dem Expressionssystem nach Anspruch 3.
5. Protein, gebildet von den Zellen nach Anspruch 4.
6. System nach Anspruch 3, dadurch **gekennzeichnet**, daß es weiterhin, angrenzend an den codierenden Teil und im Leseraster damit, eine fusionierte Nukleotidsequenz, die für ein nicht-HAV-codiertes Protein oder einen Teil davon codiert, enthält.
- 10 7. Rekombinantes Expressionssystem, umfassend einen codierenden Teil, abgeleitet von der Sequenz nach Anspruch 1, in ausführbarer Weise gebunden an eine mit dem gewünschten Wirt verträgliche Kontrollsequenz, enthaltend weiterhin stromaufwärts des codierenden Teils und getrennt durch ein Stopcodon, eine fusionierte Nukleotidsequenz, die für nicht-HAV-codiertes Protein oder einen Teil davon codiert.
- 15 8. System nach Anspruch 6 oder 7, dadurch **gekennzeichnet**, daß die fusionierte DNA-Sequenz für menschliche Superoxid-Dismutase oder einen Teil davon codiert.
- 20 9. System nach den Ansprüchen 6 oder 7, dadurch **gekennzeichnet**, daß die fusionierte DNA-Sequenz für ein nicht-HAV-Teilchen-bildendes Protein codiert.
- 25 10. Rekombinante Wirtszellen, transformiert mit dem System nach Anspruch 6 oder 7.
11. Protein, gebildet von den Zellen nach Anspruch 10.
12. System nach Anspruch 3, dadurch **gekennzeichnet**, daß der codierende Teile die Nukleotidsequenz nach Anspruch 1 zwischen den Nukleotidpositionen, ausgewählt aus der Gruppe bestehend aus 2072-3201, 1182-3201 und 734-3201, ist.
- 30 13. Antigenes Peptid, umfassend eine Sequenz, abgeleitet von der Sequenz nach Anspruch 1, in covalenter Bindung an den C-Terminus an Cystein, ausgewählt aus der Gruppe:

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-Ser-Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Thr-Phe-Pro-Cys; und

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-Ser-Thr-Cys.

14. Protein nach Anspruch 5, dadurch **gekennzeichnet**, daß das Protein ein Epitop von HAV in Kopplung an ein nicht-HAV-Teilchen-bildendes Protein, unter Bildung eines Komplexes enthält, so daß der Komplex in der Lage ist, ein Teilchen zu bilden, das gegen eine HAV-Infektion immunogen ist.
- 50 15. Protein nach Anspruch 14, dadurch **gekennzeichnet**, daß das Epitop von den Aminosäuren 445-657 oder 792-848 der HAV-Polypeptidsequenz abgeleitet ist.
- 55 16. Oligonukleotid, geeignet zur Detektion auf die Anwesenheit von HAV in einer biologischen Probe, umfassend eine DNA-Sequenz, abgeleitet von der Nukleotidsequenz nach Figur 1, wobei das Oligonukleotid ein Fragment nach Anspruch 3 ist.



17. Protein nach einem der Ansprüche 5, 11 und 13 bis 15 zur Verwendung in einem Impfstoff, der gegen das Hepatitis-A-Virus wirksam ist.
18. Expressionssystem für ein deletiertes HAV-Virion, umfassend eine Nukleotidsequenz, codierend für das P1-Precursor-Protein, wie in Figur 1 gezeigt, wobei die Nukleotidsequenz in ausführbarer Weise an geeignete Kontrollsequenzen gebunden ist und in eine Wirtszelle transformiert ist, die in der Lage ist, eine Protease zu erzeugen, die wirksam den P1-Precursor unter Erhalt eines deletierten HAV-Virions spaltet.
19. Expressionssystem nach Anspruch 18, dadurch gekennzeichnet, daß die Protease von der 3C HAV-Region der Figur 1 codiert ist.
20. Expressionssystem nach Anspruch 18, dadurch gekennzeichnet, daß das deletierte Virion von einer DNA, entsprechend den Nukleotiden 734-5767 der Sequenz nach Figur 1 codiert ist.
21. Protein, gebildet von einem Expressionssystem nach einem der Ansprüche 18 bis 20.
22. Expressionssystem nach Anspruch 3, dadurch gekennzeichnet, daß die Nukleotidsequenz für VP1, VP2 oder VP3 codiert.
23. Verfahren zur Herstellung eines Proteins, umfassend HAV-Sequenzen, dadurch gekennzeichnet, daß man Zellen nach Anspruch 4 oder Anspruch 10 oder Zellen, die ein Expressionssystem nach Anspruch 18 enthalten, kultiviert und das so erhaltene Protein gewinnt.