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3' TERMINAL SEQUENCE OF
HEPATITIS C VIRUS GENOME AND
DIAGNOSTIC AND THERAPEUTIC
USES THEREOF

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Patentee (s):	כעל (י) הפטנט:		
WASHINGTON UNIVERSITY ONE BROOKINGS DRIVE ST. LOUIS, MISSOURI, U.S.A.			

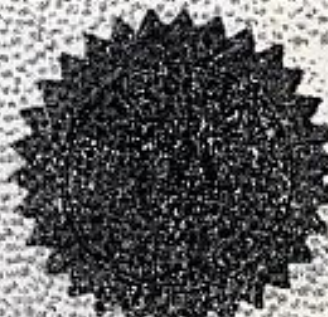
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רשם הפטנטים, המדגמים
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* Conditions of the Patent as in attachment

* תנאי הפטנט ראה כמצורף

NOVEL 3' TERMINAL SEQUENCE OF HEPATITIS C VIRUS GENOME
AND DIAGNOSTIC AND THERAPEUTIC USES THEREOF

GOVERNMENTAL SUPPORT

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TECHNICAL FIELD OF THE INVENTION

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The present invention relates generally to a novel nucleotide sequence element identified at or near the 3' terminus of the hepatitis C virus (HCV) viral genome RNA. This element is highly conserved among HCV genotypes and may be essential for HCV replication.

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BACKGROUND OF THE INVENTION

After the development of diagnostic tests for hepatitis A virus and hepatitis B virus, an additional agent, which could be experimentally transmitted to chimpanzees (Alter et al, 20 1978; Hollinger et al, 1978; Tabor et al, 1978), became recognized as the major cause of transfusion-acquired hepatitis. cDNA clones corresponding to the causative non-A non-B (NANB) hepatitis agent, called hepatitis C virus (HCV), were reported in 1989 (Choo et al, 1989). This breakthrough has led to rapid advances in diagnostics, and in our understanding of the epidemiology, pathogenesis and molecular virology of HCV 25 (see Houghton et al, 1994 for review). Evidence of HCV infection is found throughout the world and the prevalence of anti-HCV antibodies ranges from 0.4-2% in most developed countries to more than 14% in Egypt (Hibbs et al, 1993). Besides transmission via blood or blood products, or less frequently by sexual and congenital routes, sporadic cases, not associated with known risk factors, occur and account for 30 more than 40% of HCV cases (Alter et al, 1990; Mast and Alter, 1993). Infections are usually chronic (Alter et al, 1992) and clinical outcomes range from an inapparent carrier state to acute hepatitis, chronic active hepatitis, and cirrhosis which is strongly associated with the development of hepatocellular carcinoma. Although alpha IFN has been shown to be useful for the treatment of some patients with chronic HCV infections 35 (Davis et al, 1989; DiBisceglie et al, 1989) and subunit vaccines show some promise in the chimpanzee model (Choo et al, 1994), future efforts are needed to develop more effective therapies and vaccines. The considerable diversity observed among different HCV isolates (for review, see Bukh et al, 1995), the emergence of genetic variants in chronically infected individuals (Enomoto et al, 1993; Hijikata et al, 1991; Kato et al,

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1992; Kato et al, 1993; Kurosaki et al, 1993; Lesniewski et al, 1993; Ogata et al, 1991; Weiner et al, 1991; Weiner et al, 1992), and the lack of protective immunity elicited after HCV infection (Farci et al, 1992; Prince et al, 1992) present major challenges towards these goals.

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Molecular biology of HCV

Classification. Based on its genome structure and virion properties, HCV has been classified as a separate genus in the flavivirus family, which includes two other genera: the flaviviruses [such as yellow fever virus (YF)] and the animal pestiviruses [bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV)] (Francki et al, 10 1991). All members of this family have enveloped virions that contain a positive-strand RNA genome encoding all known virus-specific proteins via translation of a single long open reading frame (ORF; see below).

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Structure and physical properties of the virion. Little information is available on the structure and replication of HCV. Studies have been hampered by the lack of a cell culture system able to support efficient virus replication and the typically low titers of infectious virus present in serum. The size of infectious virus, based on filtration experiments, is between 30-80 nm (Bradley et al, 1985; He et al, 1987; Yuasa et al, 20 1991). HCV particles isolated from pooled human plasma (Takahashi et al, 1992), present in hepatocytes from infected chimpanzees, and produced in cell culture (Shimizu et al, 1994a) have been visualized (tentatively) by electron microscopy. Initial measurements of the buoyant density of infectious material in sucrose yielded a range of values, with the majority present in a low density pool of < 1.1 g/ml (Bradley et al, 25 1991). Subsequent studies have used RT/PCR to detect HCV-specific RNA as an indirect measure of potentially infectious virus present in sera from chronically infected humans or experimentally infected chimpanzees. From these studies, it has become increasingly clear that considerable heterogeneity exists between different clinical samples, and that many factors can affect the behavior of particles containing HCV 30 RNA (Hijikata et al, 1993; Thomssen et al, 1992). Such factors include association with immunoglobulins (Hijikata et al, 1993) or low density lipoprotein (Thomssen et al, 1992; Thomssen et al, 1993). In highly infectious acute phase chimpanzee serum, HCV-specific RNA is usually detected in fractions of low buoyant density (1.03-1.1 g/ml) (Carrick et al, 1992; Hijikata et al, 1993). In other samples, the presence of HCV 35 antibodies and formation of immune complexes correlate with particles of higher density and lower infectivity (Hijikata et al, 1993). Treatment of particles with chloroform, which inactivates infectivity (Bradley et al, 1983; Feinstone et al, 1983), or with

nonionic detergents, produces RNA containing particles of higher density (1.17-1.25 g/ml) believed to represent HCV nucleocapsids (Hijikata et al, 1993; Kanto et al, 1994; Miyamoto et al, 1992).

- 5 There have been many reports of varying levels of negative-sense HCV-specific RNAs in sera and plasma (see Fong et al, 1991). However, it seems unlikely that such RNAs are essential components of infectious particles since some sera with high infectivity can have low or undetectable levels of negative-strand RNA (Shimizu et al, 1993). The virion protein composition has not been rigorously determined, but putative HCV
10 structural proteins include a basic C protein and two membrane glycoproteins, E1 and E2.

HCV replication. Early events in HCV replication are poorly understood. Cellular receptors for the HCV glycoproteins have not been identified. The association of some
15 HCV particles with beta-lipoprotein and immunoglobulins raises the possibility that these host molecules may modulate virus uptake and tissue tropism. Studies examining HCV replication have been largely restricted to human patients or experimentally inoculated chimpanzees. In the chimpanzee model, HCV RNA is detected in the serum as early as
20 3 days post-inoculation and persists through the peak of serum alanine aminotransferase (ALT) levels (an indicator of liver damage) (Shimizu et al, 1990). The onset of viremia is followed by the appearance of indirect hallmarks of HCV infection of the liver. These include the appearance of a cytoplasmic antigen (Shimizu et al, 1990) and ultrastructural changes in hepatocytes such as the formation of microtubular aggregates for which HCV previously was referred to as the chloroform-sensitive "tubule forming
25 agent" or "TFA" (reviewed by Bradley, 1990). As shown by the appearance of viral antigens (Blight et al, 1993; Hiramatsu et al, 1992; Krawczynski et al, 1992; Yamada et al, 1993) and the detection of positive and negative sense RNAs (Fong et al, 1991; Gunji et al, 1994; Haruna et al, 1993; Lamas et al, 1992; Nouri Aria et al, 1993; Sherker et al, 1993; Takehara et al, 1992; Tanaka et al, 1993), hepatocytes appear to
30 be a major site of HCV replication, particularly during acute infection (Negro et al, 1992). In later stages of HCV infection the appearance of HCV-specific antibodies, the persistence or resolution of viremia, and the severity of liver disease, vary greatly both in the chimpanzee model and in human patients. Although some liver damage may occur as a direct consequence of HCV infection and cytopathogenicity, the emerging
35 consensus is that host immune responses, in particular virus-specific cytotoxic T lymphocytes, may play a more dominant role in mediating cellular damage (see Rice and Walker, 1995 for review).

It has been speculated that HCV may also replicate in extra hepatic reservoir(s), particularly in chronically infected individuals. In some cases, RT/PCR or *in situ* hybridization has shown an association of HCV RNA with peripheral blood mononuclear cells including T-cells, B-cells, and monocytes (Blight et al, 1992; Bouffard et al, 1992; 5 Gil et al, 1993; Gunji et al, 1994; Moldvay et al, 1994; Nuovo et al, 1993; Wang et al, 1992; Young et al, 1993; Yun et al, 1993; Zignego et al, 1992). Such tissue tropism could be relevant to the establishment of chronic infections and might also play a role in the association between HCV infection and certain immunological abnormalities such as mixed cryoglobulinemia (reviewed by Ferri et al, 1993), glomerulonephritis, and rare 10 non-Hodgkin's B-lymphomas (Ferri et al, 1993; Kagawa et al, 1993). However, the detection of circulating negative strand RNA in serum, the difficulty in obtaining truly strand-specific RT/PCR (Gunji et al, 1994), and the low numbers of apparently infected cells have made it difficult to obtain unambiguous evidence for replication in these tissues *in vivo*.

15 Although a cell culture system capable of efficient HCV replication has not been developed, some progress has been made. Consistent with the *in vivo* observations mentioned above, *in vitro* HCV infection and short term replication have been reported for chimpanzee and human hepatocytes (Carloni et al, 1993; Iacovacci et al, 1993; 20 Lanford et al, 1994), a human hepatoma line (Huh7; Yoo et al, 1995, see below), peripheral blood leukocytes (Muller et al, 1993), a human B-cell line expressing EBV antigens (Bertolini et al, 1993), a mouse retrovirus-infected human T-cell line (Molt4-Ma; Shimizu et al, 1992), an HTLV-1 transformed human T-cell line (MT-2; Kato et al, 1995), and fibroblasts derived from human foreskin (Zibert et al, 1995). Thus far, only 25 a small fraction of these cells appear infected. *In vitro* infectivity of different HCV inocula using a permissive subclone of the Molt4-Ma T-cell line correlates well with their *in vivo* infectivity in the chimpanzee model (Shimizu et al, 1993). This cell line has also been used to begin examining HCV binding and the possible emergence of neutralization escape mutants during chronic infection (Shimizu et al, 1994b).

30 Genome structure. Full-length or nearly full-length genome sequences of numerous HCV isolates have been reported (see Lin et al, 1994; Okamoto et al, 1994; Sakamoto et al, 1994 and citations therein). Given the considerable genetic divergence among isolates, it is clear that several major HCV genotypes are distributed throughout 35 the world (see below). Those of greatest importance in the U.S. are genotype 1, subtypes 1a and 1b. HCV genome RNAs are about 9.4 kilobases in length. The 5' NTR is 341-344 bases and is the most conserved RNA sequence element in the HCV

genome. The length of the long ORF varies slightly among isolates, encoding polyproteins of 3010, 3011 or 3033 amino acids. The reported 3' NTR structures show considerable diversity both in composition and length (28-42 bases), and appear to terminate with poly (U) (for examples, see Chen et al, 1992; Okamoto et al, 1991; Tokita et al, 1994) except in one case (HCV-1, type 1a) which appears contain a 3' terminal poly (A) tract (Han et al, 1991).

Translation and proteolytic processing. Several studies have used cell-free translation and transient expression in cell culture to examine the role of the 5' NTR in translation initiation (Fukushi et al, 1994; Tsukiyama-Kohara et al, 1992; Wang et al, 1993; Yoo et al, 1992). This highly conserved sequence contains multiple short AUG-initiated ORFs and shows significant homology with the 5' NTR region of pestiviruses (Bukh et al, 1992; Han et al, 1991). A series of stem-loop structures have been proposed on the basis of computer modeling and sensitivity to digestion by different ribonucleases (Brown et al, 1992; Tsukiyama-Kohara et al, 1992). Although still controversial (see Wang et al, 1993; Yoo et al, 1992), the results from several groups indicate that this element functions as an internal ribosome entry site (IRES) allowing efficient translation initiation at the first AUG of the long ORF (Fukushi et al, 1994; Tsukiyama-Kohara et al, 1992; Wang et al, 1993). Some of the predicted features of the HCV and pestivirus IRES elements are similar to one another (Brown et al, 1992). It has been proposed that the 5' terminal hairpin structure and the short ORFs may function to downregulate translation (Yoo et al, 1992). The ability of this element to function as an IRES suggests that HCV genome RNAs may lack a 5' cap structure.

The organization and processing of the HCV polyprotein appears to be most similar to that of the pestiviruses. At least 10 polypeptides have been identified and the order of these cleavage products in the polyprotein is NH₂-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Proteolytic processing is mediated by host signal peptidase and two HCV-encoded proteinases, the NS2-3 autoproteinase and the NS3-4A serine proteinase. C is a basic protein believed to be the viral core or capsid protein; E1 and E2 are putative virion envelope glycoproteins; p7 is a hydrophobic protein of unknown function that is inefficiently cleaved from the E2 glycoprotein (Lin et al, 1994; Mizushima et al, 1994; Selby et al, 1994), and NS2-NS5B are likely nonstructural (NS) proteins which function in viral RNA replication complexes. In particular, besides its N-terminal serine proteinase domain, NS3 contains motifs characteristic of RNA helicases and has been shown to possess an RNA-stimulated NTPase activity (Suzich et