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Hepatitis C Virus-Specific DNA Sequences in Human DNA: Differentiation by Means of Restriction Enzyme Analysis at the DNA Level in Healthy, Anti-HCV-Negative Individuals

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This study aimed to look for further HCV-specific sequences at the DNA level of healthy, HCV-negative individuals. Here, the sequence section from nt 57 to nt 328 within the 5'-NCR was assayed. Different combinations of primers were used in the nested PCR without a preceding reverse transcriptase step, resulting in fragments of the expected molecular weight size and also shorter and longer ones. It shows that the major part of the 5'-non-coding region (5'-NCR) of the hepatitis C virus genome is present in the DNA fraction from peripheral blood mononuclear cells (PBMCs) of healthy, anti-HCV-negative individuals tested. Furthermore we designed experiments to prove the specificity of these findings, by using restriction enzymes for digest assays of the target DNA before PCR (pre-PCR digest) and of the products after PCR (post-PCR digest). In conclusion, our study indicates that the main part of the internal ribosome entry site (IRES) structure of HCV at least is contained in the DNA of the individuals tested.

Key words: HCV; HCV-DNA; Anti-HCV-negative individuals.

Introduction

At least 90% of hepatitis C virus (HCV)-infected patients continue to carry the virus indefinitely as shown by the assessment of HCV RNA (1). Persistent hepatitis C often remains silent for many years (2). The probability of hepatocellular carcinoma development also arises (3, 4). The mechanisms of HCV persistence (HCV-RNA viremia) as well as the pathogenetic mechanism of HCV induced liver damage are presently unknown (5).

The following must be considered concerning the HCV-induced diseases: although virus-like particles could be visualized under particular conditions (6) there remain dividing opinions over the issues of extrahepatic and extracellular hepatitis C virus (HCV) particles, which should correspond to sometimes extremely high concentrations of HCV-RNA in serum or plasma. HCV is accepted to be associated with autoimmune disorders of various clinical manifestations, e.g. cryoglobulinemia and certain kinds of autoimmune hepatitis. HCV sequences at the DNA level could provide a rationale for research to seek a more direct involvement of HCV in these diseases. In continuing investigations on findings of HCV-specific sequences in the peripheral blood mononuclear cells (PBMC) DNA fraction of both patients suffering from hepatitis C and healthy, anti-HCV-negative individuals, we extended the range of the sequence section that spanned 81 base pairs of the 5'-non-coding region (5'-NCR) (7). Here, we have focused only on the 5'-NCR, now encompassing 256 of its 341 base pairs.

Materials and Methods

Sources for DNA and RNA extraction

Seven (3 females, 4 males) healthy, anti-HCV-negative individuals were studied, with no history of blood transfusion, no i.v. drug use, one of them is a long-term blood donor. These individuals have never suffered from any kind of acute hepatitis. In enzyme immunoassays for hepatitis Bs-antigen: all individuals were 'not reactive'; the results of enzyme immunoassays for anti-HBc showed 2/7 reactive, 5/7 not reactive (Enzygnost, Dade-Behring, Marburg, Germany). In the past, they had regular medical routine examinations without any records. The anti-HCV EIA (Pasteur, Freiburg, Germany, or Abbott, Wiesbaden, Germany) and Matrix supplementary assay (Abbott) were performed according to the manufacturer's instructions. Aspartate aminotransferase/GOT, E.C. 2.6.1.1; alanine aminotransferase/ALT(GPT), E.C. 2.6.1.2; gamma glutamyl transpeptidase/γGT, E.C. 2.3.2.2 in the samples were within the normal range. All samples were tested for HCV-RNA, and were negative (not detectable) in all but one cases; this individual showed a transient positive result for HCV-RNA. However, a blood sample drawn four months later revealed a negative (not detectable) result. HCV-RNA determination was conducted with the HCV-RNA Amplicor assay (Hoffmann La-Roche, Grenzach-Wyhlen, Germany).

Isolation of PBMCs: ten ml venous blood was drawn and immediately adjusted with EDTA-Na to give a final concentration of 5 mM. The further handling was as described previously (7).

HeLa (cervical carcinoma), and MT2 (T-cell lines) cells were taken from continuous cell culture passages. Cell culture passages were performed according to standard procedures. Protocols for extraction of DNA, PCR, and hybridization were the same as described for PBMCs.

Extraction of DNA

The nucleic acid extraction kit of Boehringer (High Pure PCR Template Preparation Kit, Kat. No. 1796828, Boehringer,

Mannheim, Germany) was used. The nucleic acid extract was always treated with RNase (DNase free, Boehringer, Mannheim), in a final concentration of 20 µg/ml. After treatment, the RNase was extracted twice with phenol/chloroform, the DNA dissolved in Tris/HCl 10 mmol/l, EDTA 1 mmol/l, pH 8.0.

Polymerase chain reaction (PCR)

Oligonucleotides: HCV-specific primers were synthesized by MWG (Ebersberg, Germany):

AE1 : 5'-ACTGTCTTCACGCAGAAAGCGTCTAGCCAT;
AE2: 5'-CGAGACCTCC-CGGGGCACTCGCAAGCACCC;
AI1: 5'-ACGCAGAAAGCGTCTAGCCATGG-CGTTAGT;
AI2: 5'-TCCCGGGGCACTCGCAAGCACCCTATCAGG;
PTNC-E1: 5'-CGTTAGTATGAGTGTCGTGC;
PTNC-E2: 5'-CGGTGTACTCACCGGTTCC;
PTNC-I1: 5'-AGTGTCGTGCAGCCTCCAGG;
PTNC-I2: 5'-CGGTTCCGCAGA-CCACTATG;
PTNCP: 5'-ATGGCT-CTCCCGGGAGGGGG;
YE2: 5'-AACACTACTCGGCTAGCAGT.

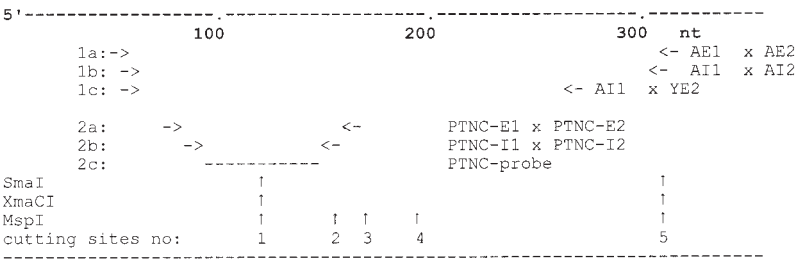
They were chosen from the 5'-non-coding region (5'-NCR) of the HCV genome according to published data (Figure 1). Both reaction mixture and PCR methodology were essentially the same as outlined previously (7). Direct PCR (dPCR) refers to PCR without any reverse transcriptase (RT) step involved.

To further prove the specificity of priming, the hot start protocol for PCR was applied in selected cases. The band pattern after PCR and gel electrophoresis remained unchanged. Finally, the temperature of the annealing step of the PCR was adjusted up to 60 °C (instead of 56 °C), but again the band patterns after gel electrophoresis remained the same. The bands present after dPCR and gel electrophoresis proved to be specific for a DNA target. The results after dPCR were sensitive for DNase treatment but were not influenced by RNase treatment of the cell extracts.

Enzymatic analysis

The restriction enzymes SmaI, XmaCI, and MspI (Boehringer, Mannheim, Germany) were used according to the manufacturer's instructions. Restriction enzyme analysis (REA) was performed on the original (target) DNA after extraction from PBMCs, that is, *before* PCR (pre-PCR digest) – and in selected cases with the *products after* PCR (post-PCR digest). DNase and RNase challenge of the human target DNA: DNase, 20 units/50 µl final concentration (RNase free, Boehringer; EC 3.1.21.1) and RNase (DNase free, Boehringer; EC 3.1.27.1), in a final concentration of 20 µg/ml were applied according to the manufacturer's instructions.

A Enlarged section of the 5'-NCR of the HCV with location of primers and restriction enzymes used.



↑: broadly marks the cutting sites for SmaI, XmaCI 'CCC↑GGG', and MspI 'C↑CGG', see part B for details; numbers give the location of cutting sites according to the prototype HCV strain (8); 1: 130/131 respectively 129/130; 2: 157/158 ; 3: 168/169; 4:186/187; 5: 317/318, respectively 316/317.

B Restriction enzymes used and their 'cutting sites':

Restriction enzyme	Sensitive cutting site	Resistant against cutting	Cutting site = nt number, according to prototype HCV (8)
SmaI	CC°C ↑GGG	C*C°C*↑ G G G	130/131, 317/318
XmaCI	CC°C° ↑GGG	C*C°C° ↑ G G G	130/131, 317/318
Msp1	C↑C°GG	C* ↑ C°G G	129/130, 157/158, 168/169, 186/187, 316/317

°: cutting is not influenced by methylation = 5-methylcytosine;
*: cutting is inhibited if 5-methylcytosine is present.

Fig. 1 Hepatitis C-virus: 5'-non-coding region (5'-NCR) of the genome with location of primers, probes, and cutting sites of restriction enzymes used (SmaI, XmaCI, and MspI).
A The 5'-non-coding region (5'-NCR) and primers used; in brackets the nucleotide number is given according to the prototype HCV strain described by Choo *et al.* (8) (EMBL, Heidelberg). References are given in parenthesis. Set 1a: AE1 [57-86], AE2 [299-328], AI1 [66-95], AI2 [292-321] (9); set 2a: PTNC-E1 [89-108], PTNC-E2 [152-170], set 2b: PTNC-I1 [99-118], PTNC-I2 [139-158], PTNC-P = digoxigenin-labeled probe [122-141] (10); YE2 [246-265] (11). E.: external, -I.: internal, -.1: sense, -.2: antisense; < >arrows indicate broadly the start position of primers, '↑' arrows indicate broadly the cutting position of the restriction enzymes used here.
B The detailed cutting sites for the restriction enzymes used. '*' or '°' mark the sites of methylated cytosine(s) that do or do not influence the activity of the restriction enzymes used (12).

Sequence analysis

Sequencing was performed by MWG (Ebersbach, Germany) after cloning the fragments into the TA cloning vector pCR 2.1 (Invitrogen, Leek, Netherlands). Both sense and antisense strands have been sequenced throughout.

Results

In continuing our previous investigations on HCV-DNA-specific sequences in human DNA of PBMCs in the healthy, anti-HCV-negative individuals (7), we first extended the range of sequences screened so far, and then tried to differentiate the sequences found at the target DNA level before PCR (pre-PCR assay).

In the present study, we have focused in particular on the 5'-NCR of the HCV genome. Up to now, the sequence region assayed was from nt 89 to nt 170, the PTNC region, according to the primers used for nested PCR (10). The extended area used here ranges from nt 57 to nt 328 (primers AE1&2 and AI1&2, Figure 1A) encompassing the PTNC region.

After PCR with the target DNA using the AE & AI primers the products show fragments at the expected position of 256 base pairs throughout (Table 1). The hybridization with the PTNC-probe showed positive signals in 2/4 tested individuals. The sequences of the hybridization positive samples show a high degree of homology with the reference strain of HCV (Figure 5; br-d3r, a HCV-positive patient for comparison, and bo-d1r). Compared with the HCV reference sequence, they reveal substitutions at the same location as well as at different positions. The fragments of the individuals without a signal after hybridization consist of sequences with only short stretches of HCV-specific nucleotides present (sequences not shown here[1]). Fragments generated from HeLa-DNA or from MT2-DNA do not show signals after hybridization. HeLa-DNA and MT2-DNA have been included here as a check against contamination.

The reason for the generation of two kinds of fragments containing high (>98 %) or low (about 16 %) homology with HCV-specific sequences may be the presence of at least two locations pre-selected and amplifiable by the 1a and 1b primer sets (see below, Figure 4). Therefore, the inner anti-sense primer AI2 of the set 1b was replaced by YE2 (i.e., set 1c, Figure 1A), corresponding to a 200 base pair fragment (11). It is located within the sequence region framed by the 1b primer set. Furthermore, the number of samples from healthy, anti-HCV-negative individuals was increased. Again, the outcome after PCR shows differences between individuals: fragments of the expected size are present in 5/7 samples, longer than expected fragments in 2/7, and an additional one shorter than the expected fragment in 1/7.The fragments show positive signals after hybridization with the PTNC-probe in 3/7 individuals (Table 1).

We further aimed to differentiate the HCV-specific regions at the target DNA level before PCR. The experimental procedure was to digest the original target DNA

[1]) In this manuscript we have focused only on fragments in the range of the expected molecular size and with high homology (>98 %) to the HCV reference sequence (8). The fragments showing low homology to HCV are not discussed here.

Tab. 1 Results after direct PCR with DNA from healthy, anti-HCV-negative individuals.

Nested PCR 1. round: 2. round:	Group I primer sets: 1a: AE-1/2 2b. PTNC-I-1/2	Group II primer sets: 2a: PTNC-E-1/2 2b. PTNC-I-1/2	Group III primer sets: 1a: AE-1/2 1b: AI-1/2	Group IV primer sets: 1a: AE-1/2 1c: AI-1/YE-2
Number of individuals tested	4	7	4	7
	Fragments of the expected size (60 bp): pos. / neg.	Fragments of the expected size (60 bp): pos. / neg.	Fragments of the expected size (256 bp): pos. / neg.	Fragments of the expected size (200 bp): pos. / neg.
After gel electrophoresis	4 / 0	7 / 0	4 / 0	5(#) / 2(#)
Signal after hybridization with 'PTNC-P' probe	4 / 0	7 / 0	2 / 2	3 / 4(#)
	res./sens.	res./sens.	res./sens.	res./sens.
Target DNA upon pre-PCR SmaI digest assay	2 / 2	7* / 0	2 / 2	n.d.

#, this includes those with fragments of longer or shorter than the expected size (fragments of longer than the expected size are not discussed here); *, one with only partly digest; 'pos.', present; 'neg.', not present; 'res.', resistant against SmaI; 'sens.', sensitive for SmaI; n.d., not done; bp, base pairs.

with restriction enzymes before set-up of the PCR (pre-PCR digest), and to use different primer sets. The PTNC region and the overlapping area of the AE2/AI2 primers both contain the CCCGGG sequence, which in turn harbors the cutting site for the restriction enzymes SmaI, XmaCI, and MspI. Therefore, these restriction enzymes were selected as they recognize the same cutting site CCCGGG (SmaI and XmaCI) and CCGG (MspI). These enzymes can be inhibited from cutting the CCCGGG respectively CCGG sequence depending on the location of methyl-cytosine within it (Figure 1B).

After digestion with SmaI of the target DNA pre-PCR, the *result after PCR* is different (Figure 2). When using the primer combination set 1a and set 2b (the size of fragments after the second round PCR is 60 base pairs), the pre-PCR digest with SmaI gave no products after PCR for both individuals (lanes 3 and 7, left half of Figure 2). However, when only the 2a and 2b primer sets for the PTNC region (the size of fragments is 60 base pairs, Figure 1A) were applied with the DNA pretreated by SmaI, no digestion took place at the target DNA level (lanes 3 and 7, right half of Figure 2): this follows, because after PCR the fragments of expected size are present, although the PTNC region containing the cutting site for SmaI is present as shown by positive signals after hybridization with the PTNC-P probe (set 2c, result not shown here) and the sequences we derived are consistent with its presence (see sequence dbo-pt1u and dni-pt1u, Figure 5). Therefore, we think of the CCCGGG sequence within the PTNC site to be resistant against SmaI.

We then extended the pre-PCR digest assays with two more restriction enzymes. The DNA of two selected individuals (Figures 3 A, B) was subjected to digestion with SmaI, XmaCI, and MspI (pre-PCR digest). Furthermore, two different combinations of primers were used: the first round was set up with different sets of external primers. However, during the second [nested] round, the internal set of primers remained the same (set 2b = PTNC-I1/2) (see legend of Figure 3). When starting with the AE1/2- primers (left half of Figure 3), SmaI and XmaCI were able to make a digestion so as to result in no products after PCR. However, when using MspI, the target DNA turned out to be resistant for both individuals (lane 7, left half of Figure 3), that is, the PCR

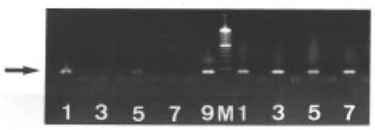


Fig. 2 Gel electrophoresis after direct PCR. Left half of 'M' no. 1 - 9, 1st round PCR: AE1 & E2, 2nd round PCR: PTNC-I1 & I2; right half of 'M' no. 1 – 7, 1st round PCR: PTNC-E1 & E2, 2nd round PCR: PTNC I1 & I2; individual 1: lanes 1 (undigested) and 3 (pre-PCR digest with SmaI); individual 2: lanes 5 (undigested) and 7 (pre-PCR digest with SmaI); both individuals: healthy, anti-HCV-negative; lane 9: positive control for fragment size (cloned fragment from a HCV-positive patient covering nucleotides 66 to 321 (7)), not treated with restriction enzyme; lanes 2, 4, 6, 8: blanks; arrow (→): marks the size of expected fragment, i.e., 60 base pairs; M: molecular weight marker, 100 base pair ladder.

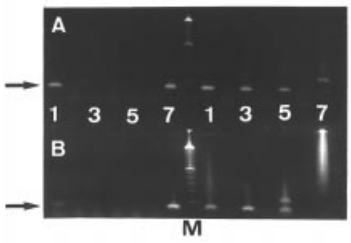


Fig. 3 Gel electrophoresis after direct PCR with primers: left of 'M', 1st round PCR: AE1/2, 2nd round PCR: PTNC-I1/2; right of 'M', 1st round PCR: PTNC-E1/2, 2nd round PCR: PTNC-I1/2. Two healthy, anti-HCV-negative individuals A, B: lane 1: original target DNA undigested; lane 3: original target DNA digested with SmaI before PCR was set up (pre-PCR digest); lane 5: pre-PCR digest with XmaCI, lane 7: pre-PCR digest with Msp1, respectively; lanes 2, 4, 6: blanks; arrows (→) mark the size of expected fragments, i.e., 60 base pairs; M: molecular weight marker, 100 base pair ladder.

did generate fragments of the expected size. When using only the external and internal primers of the PTNC-region (i.e., set 2a and set 2b) no pre-PCR digestion occurred with SmaI and XmaCI. This follows, because after PCR the fragments of the expected size are present (lane 3 and 5, right half of Figure 3). However, with individual B, along with the fragment of the expected size, the XmaCI pre-PCR digest generated a longer than expected fragment of about 126 base pairs (lane 5, right half of Figure 3). The sequence of this shows one additional PTNC-E2 sequence located before and after a sequence of unknown origin (database search failed to detect a similar sequence) as well as one additional PTNC-I1 sequence (sequence not shown here). The MspI pre-PCR digest resulted in a single fragment longer (125 bp) than expected for individual A (the structure of this fragment is similar to that mentioned before) (lane 7, right half of Figure 3) and a smear for individual B.

We additionally analyzed the products after PCR by means of digestion with a selected restriction enzyme, SmaI (post-PCR digest), that is, a combined pre- and post-PCR digest protocol. The reason for this is as follows: resistance against cutting by SmaI pre-PCR could be caused by a methyl-cytosine at a certain position of the CCCGGG recognition site (Figure 1B). During PCR, the methylated cytosines should become converted into un-methylated cytosines by incorporation of regular cytosines, thereby rendering the cutting site sensitive to SmaI. Figures 4A and 4C show, for two selected individuals, the results of SmaI digests of both the target DNA pre-PCR, and of the post-PCR products. Since SmaI is now able to digest the products generated by PCR (post-PCR digest), the SmaI resistant cutting site of the undigested target DNA (part A: lanes 1, for primer combinations I, II and III, and part C: lanes 1-/I and 1-/III), must have been converted into a SmaI-sensitive cutting site during PCR (part A: lanes 2-/I, 2-/II, and 2-/III, part C: lanes 2-/I and 2-/III). When using the primer combination III, the original untreated target sequence results in just one visible band at the expected position (lanes 1-/III, A, C). However, the SmaI post-PCR digest is incomplete, that is, it results in a shorter fragment of 188

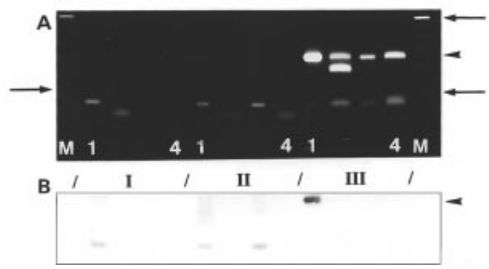


Fig. 4A Gel electrophoresis, results after digestion assays of the original target DNA before (pre-PCR) and after PCR (post-PCR) with different primer combinations. Restriction enzyme SmaI, one selected individual (anti-HCV-negative, transaminase within the normal range, HCV-RNA transiently reactive). Primer combinations used: I: 1st round primer: AE1 x AE2 (set 1a), 2nd round primer: PTNC-I1 x PTNC-I2 (set 2b), size of expected fragment 60 base pairs; II: 1st round primer: PTNC-E1 x PTNC E2 (set 2a), 2nd round primer: PTNC-I1 x PTNC-I2 (set 2b), size of expected fragment, 60 base pairs; III: 1st round primer: AE1 x AE2 (set 1a), 2nd round primer: AI1 x AI2 (set 1b), size of expected fragment is 256 base pairs.
Lanes 1- /I, -/II, -/III: undigested, original target DNA;
lanes 2- /I, -/II, -/III: digests of products from lanes 1-/I, -/II, -/III (post-PCR digest);
lanes 3- /I, -/II, -/III: digest of target DNA before PCR (pre-PCR digest);
lanes 4- /I, -/II, -/III: digests of products from lanes 3-/I, -/II, -/III (post-PCR digest) .
M: molecular weight marker; arrows (→ ←, and ←): base pair ladder, 100 and 600 base pairs; right margin arrows (≺): fragments of the expected molecular weight mass (256 base pairs) when amplified with 1a and 1b primer sets, group III.

Fig. 4B Results after hybridization of the products shown in the gel of Figure 4A. Hybridization was done with the probe PTNC-P (Figure 1A).

base pairs (see sequence bo-aid4r, Figure 5), and a very short fragment of about 65 base pairs that disappears in the range of the primer/dimer complexes. This should occur owing to the location of the cutting site belonging to a fragment with HCV-specific sequences, as shown by a positive signal after hybridization prior to the digest (lane 1-/III, part B). Also, an additional fragment of the size of about 256 base pairs indicates 'not sensitive for SmaI', i.e., that no cutting site(s) are present. Therefore, the band hybridizable at the expected position (256 base pairs) shown for lane 1-/III (part B for individual A only) should contain at least two kinds of fragments with quite a different composition: one of high homology with the respective HCV sequence, and the other with a sequence of only a low degree of homology (about 16%, Table 1). This also followed with digested DNA pre-PCR in combination with 1a and 1b primers (lanes 3-/III, pre-PCR, Figure 4 A, C) that did not create a product that was hybridizable; that is, the cutting site within the HCV-specific target DNA had been destroyed by pre-PCR digest with SmaI, but not the 'non-specific' one that does not harbor a PTNC site. Therefore, the SmaI resistant fragment should be due to either a methylation or a mutated nucleotide within the CCCGGG cutting site of the AE2 primer annealing site of the target DNA. This issue remains to be determined.

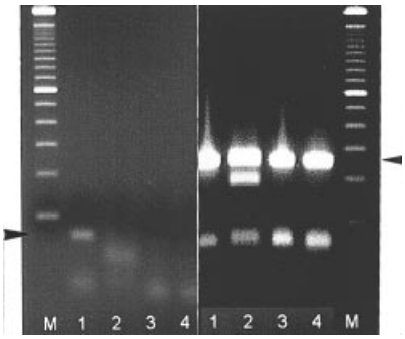


Fig. 4C Same as in Figure 4A, but here the results after direct PCR and gel electrophoresis for another healthy, anti-HCV-negative individual are shown; the HCV-RNA remained negative (not detectable) throughout the time of enrollment and 5 months later. Here, primer combinations I (left half) and III (right half) have been used. Restriction enzyme SmaI only.
Lanes 1-/I, -/III: undigested, original target DNA;
lanes 2-/I, -/III: digests of products from lanes 1-/I, -/III (post-PCR digest);
lanes 3-/I, -/III: digests of target DNA before PCR (pre-PCR digest);
lanes 4-/I, -/III: digests of products from lanes 3-/I, -/III (post-PCR digest) .
M: 100 base pair ladder; left margin arrow (≻): fragment of the expected molecular weight mass (60 base pairs) when amplified with 1a and 2b primer sets, group I; right margin arrow (≺): fragments of the expected molecular weight mass (256 base pairs) when amplified with 1a and 1b primer sets, group III.

Discussion

The present study aimed to look for further HCV-specific sequences in the DNA of healthy, anti-HCV-negative individuals. Here, we have extended the range within the 5'-NCR of HCV using primers encompassing nt 57 to nt 328 – an area that includes the PTNC site (7, 10). A pre-PCR digest protocol with restriction enzymes for the target DNA was included to test the specificity of the amplified sequences.

The results after nested PCR demonstrate that depending on the combination of primers used fragments of the expected molecular weight are generated and in some cases both longer than expected and shorter than expected ones are present. The hybridization of these products revealed that not all of them do contain the sequence detectable by the PTNC-probe (nt 122 to nt 141), even in fragments of the expected size. The sequences of those fragments giving no signal after hybridization with the PTNC-probe contain a low degree of homology (about 16%) with HCV. However, the homology of the corresponding sequences annealing with the primers must be high since challenging under higher stringency conditions during PCR (annealing temperature up to 60 °C) resulted in fragments of the same size. This must be true at least for the 3'-end of the primers, where the Taq polymerase starts.

Can the target sequences be specified, distinguished? Further combinations of different primer sets for nested PCR (Figures 3 and 4), and the additional digestion of target DNA with restriction enzymes before

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                100       110       120       130       140       150
hcvchoo     ATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTG
br-d3r      .......t..a.......-.y.................................
bo-d1r      .......ct..a.......-.................................
bo-aid4r                                        ...................
dbo-pt1u                           ...................
dni-pt1u                           ...................

                       160       170       180       190       200
hcvchoo          CGGAACCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTG
br-d3r           ........................c...g.....................
bo-d1r           ......t.................c...g.....................
bo-aid4r         ......t.................c...g.....................

                        210       220       230       240       250
hcvchoo          GATCAA_CCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGC
br-d3r           ..a...a....t........c.aca.........c.................
bo-d1r           ..a...a.............c..ca.......t...................
bo-aid4r         ..a...a.............c..ca.......t...................

                       260       270       280       290
hcvchoo          TAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTG
br-d3r           ..........c..............................
bo-d1r           ...............................a.........
bo-aid4r         ...............................a.........
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Fig. 5 Alignment of nucleotide sequences of fragments after direct PCR without the primer sequences. 'hcvchoo', sequence for reference of index HCV including its numbering (8); 'br-d3r', patient with chronic hepatitis C. The sequence of this patient has been included here only for the sake of comparison; 'bo-d1r', healthy, anti-HCV-negative individual, see Table 1 (both amplified with 1a and 1b primer sets); 'bo-aid4r', healthy, anti-HCV-negative individual, see Figure 4A, lane 2/III, fragment at about 188 base pairs, 1a and 1b primer sets and post-PCR digest with SmaI; 'dbo-pt1u' and 'dni-pt1u' both healthy, anti-HCV-negative individuals, 2a and 2b primer sets, lanes 3 and 7, right half of Figure 2. Upper case code in 'hcvchoo' indicates the index HCV sequence. Only the 'hcvchoo' sequence of the inner sense (downstream) primer PTNC-I1 and the inner anti-sense (upstream) primer PTNC-I2 is shown. The nucleotides different from the index HCV sequence are shown in lower case letters, periods '.' represent homologous nucleotides, '–' represent deletions.

use in PCR (pre-PCR digest) and of the products after PCR (post-PCR digest) reveal that if the nested PCR was performed with sets 2a and 2b (PTNC primers only), the pre-PCR digestion assay of the target DNA with SmaI and XmaCI had no effect on the outcome after PCR, indicating resistance against both these enzymes before the PCR was done. Such an inhibition could be due to, for example, methylations present at the cytosines no 1 and no 3 within the $C^1C^2C^3GGG$ recognition site (12). During amplification, the methylated cytosines become replaced by regular cytosines, now rendering the respective cutting site sensitive to the same restriction enzymes in the post-PCR digest. The same phenomenon, rendering the target sequence sensitive during PCR, was observed when the restriction enzyme MspI, cutting within the same CCCGGG site, was applied following exactly the same pre-PCR and post-PCR digest protocol that was used with SmaI. If the target DNA is resistant against digest with MspI before PCR, the amplificates after PCR are sensitive upon digest with MspI. Digests with HpaII, an isoschizomer of MspI, revealing 'post-PCR sensitivity' have been done previously (7). Furthermore, it follows that the resistance against SmaI, XmaCI and MspI of this recognition site is not due to a nucleotide substitution. This could be shown by sequence analysis (sequences not shown). The most probable explanation for the occurrence of fragments of longer than the expected size after a digestion of target DNA by MspI pre-PCR (part A, lane 7, right half, Figure 3) and XmaCI (part B, lane 5, right half, Figure 3) is that it points to a structure of this sequence section to be present like repeated section(s) at least twice that may harbor a resistant and a sensitive cutting site respectively for the restriction enzymes used.

The preselection with the AE1 & AE2 primer pair (set 1a for the 1st round of PCR) for the PTNC-I1 & -I2 sequence section (set 2b for the 2nd round of PCR) disclosed a different sensitivity of the two CCCGGG recognition sites, the one within the PTNC region, the other within the sequence annealing region for the outer antisense AE2 primer: the digest of the target DNA pre-PCR with SmaI respectively XmaCI turned out to be effective in that no products after PCR are present. This is true, although the CCCGGG site within the PTNC region in each case tested so far proved to be resistant against SmaI and XmaCI. Therefore, we conclude that the CCCGGG recognition site within the annealing site for the AE2 primer sequence is sensitive for both these restriction enzymes, thus not allowing this target to be amplified. Obviously, this might be due to the un-methylated cytosines no 1 and no 3 of the $C^1C^2C^3GGG$ (Figure 1B) within the target DNA sequence for the AE2 primer (nt 315 to 320, Figure 1A). The different pattern of methylation at cutting sites for the restriction enzymes used at the original target DNA level turned out to be stable over weeks, in some cases which have been tested so far also stable for months.

The point at issue here is that a) combining pre-PCR digestion assays of the target DNA with selected restriction enzymes and b) PCR with various combination of primers reveals:

1) The target sequences can be distinguished with different restriction enzymes pre-PCR in general, and furthermore, with regard to the methylation pattern of the cutting sites.

2) The additional post-PCR digest assay further enables to locate sequences that may contribute to regulatory functions within IRES structures.

3) This procedure can be used as a further check for specificity.

4) The DNA of the human individuals tested contains a 272 base pair section of the 5'-NCR of the HCV encompassing most of the sequence belonging to the IRES structure of the HCV.

The findings of parts of the sequence section of the HCV genome contained in the human DNA anti-HCV-negative, healthy individuals bring up the question about the possible function and nature of such HCV-specific DNA sequences. IRES harboring sequences of a human mRNA are known, e.g., those pertaining to the regulation of the heavy chain of immunoglobulins (13). The 5'-NCR of HCV contains the potential to form an IRES structure (14, 15). Naturally (?) and transiently occurring transcripts from HCV-IRES-specific DNA sections could happen to be detected without any clinical signs in healthy, anti-HCV-negative individuals as revealed for such an individual under study here (Figure 4A). If only the IRES as part of the 5'-NCR is concerned, no anti-HCV can be elicited under such circumstances. Methylation sites at the DNA level are known to be responsible for regulation at the transcriptional level (16). Therefore, the conclusion described here could point to a mechanism at the DNA level that could control by preventing or supplying RNA sequences containing such an IRES structure. Previously described RNA transcripts of longer than the expected molecular size in HCV-positive patients provide hints for such transcripts originating from the human DNA (17, 18). Very tentatively, the following issues should be considered in relation to the HCV-specific sequences within the human DNA:

1) Whether they enable to establish a process of sustained autonomous replication, that is, to set up a replicative process by its own after de-silencing by an unknown mechanism.

2) Could the HCV-specific sequences present in the human genome serve by way of transcripts as a supply for extraneous, i.e., incoming RNA sequences to be completed, or modified, by means of RNA recombination (19)? This requires additional knowledge as to what extent the human DNA contains further HCV-specific sequences.

As to the possible origin, two situations should be considered: do the HCV-specific sequences detected and specified at an individual DNA level represent constituents of the human genome in the meaning of endogenous sequences, or is there a mechanism responsible by way of an endogenous reverse transcriptase step? The latter should be comparable with the one shown for the lymphocytic choriomeningitis virus (LCM) (20). However, this does not seem to be a realistic alternative, since the healthy, anti-HCV-negative individuals investigated here do not show any indications of a previous, or ongoing, infection by HCV. It rather seems that the HCV-specific DNA sequences found and analyzed here belong to naturally occurring sequences in the human DNA assayed. However, regarding our previous results where we found the PTNC-sequence section in 14 out of 15 (including those reported here) tested healthy, anti-HCV-negative individuals (7), it seems probable that these findings reflect a phenomenon of a more fundamental significance. Hence, this could be similar to a situation comparable to that of the cellular homologue for the hepatitis delta antigen (21) or the endogenous retroviral related sequences, HERV (22). Then, by carefully extending these findings, it would consistently refer to an old evolutionary source as already intimated by others (14, 23).

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