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The GOR gene product cannot cross-react with hepatitis C virus in humans

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SUMMARY

GOR (GOR47–1) is an epitope thought to be a host-derived antigen cross-reactive with hepatitis C virus (HCV) since it was isolated from a cDNA library of host animals reactive with sera of HCV-positive patients. An enzyme immunosorbent assay (ELISA) using this epitope as antigen is of sufficient sensitivity and specificity for screening patients with HCV. However, the relationship between GOR47–1 epitope and autoimmune phenomena associated with HCV infection or autoimmune hepatitis is controversial. Here we isolated the human GOR gene and found that the GOR47–1 epitope was not translated in humans due to a single base replacement from chimpanzee. Furthermore, we found some patients who had antibodies against another epitope, which is translated (GOR1–125) in humans, although there was no correlation between the existence of anti-GOR47–1 or anti-GOR1–125 Ab and autoimmune phenomena. Serum IgG levels did not influence the titres of these antibodies. Taken together with the results of several other studies, our finding that the GOR47–1 epitope cannot be translated into a protein suggests that there is little relationship between autoimmunity and the GOR gene product in human beings. We also discuss here the possible mechanism of cross-reactivity between HCV and the GOR gene product.

Keywords GOR HCV autoantigen autoimmunity

INTRODUCTION

In the process of investigating the causal virus of non-A non-B hepatitis (NANB hepatitis), Mishiro *et al*. [1] identified the GOR47–1 antigenic epitope in the serum of NANB hepatitis-infected chimpanzee. Subsequently, an enzyme immunosorbent assay (ELISA) using synthetic partial GOR peptide (GOR47–1 epitope) as antigen, became available to detect patients infected with hepatitis C virus (HCV), which was more sensitive than the first generation antibody system [2]. Later, more sensitive detection systems, designated second or third antibody, were established and the relevance of the GOR47–1 peptide to HCV infection in this context has diminished. It is of interest that a peptide derived from a host animal could react to anti-HCV Ab, as autoimmune diseases are potentially triggered by viral infections. Moreover, it is well known that HCV infection can sometimes be complicated by autoimmune disorders, such as cryoglobulinemia [3,4], skin diseases [5,6], sialoadenitis [7], and autoimmune hepatitis [8]. Despite several studies, a significant relationship between anti-GOR47–1 Ab and autoimmunity has not been substantiated. The role of anti-GOR47–1 Ab in autoimmune processes is therefore controversial.

Recently, we independently isolated the human counterpart of the GOR gene transcript from cDNA of a mononuclear cell line using RNA arbitrary primed-polymerase chain reaction (RAP-PCR) differential display technique [9]. Although the isolated human GOR gene was highly conserved with that of chimpanzee, which is compatible with the genetic similarity between human and chimpanzee, we found a critical difference between the two species. That is, the region corresponding to the GOR47–1 epitope could not be translated in humans because of a single base replacement. Considering this, we examined the antigenicity of the region that can be translated in humans, in HCV-infected and other patients to determine whether the GOR gene product can cross-react with anti-HCV Ab and be relevant to autoimmunity. Although some patients expressed antibodies to recombinant partial GOR protein (GOR1–125 epitope), which is translated in humans, there was no association with HCV infection. Most HCV patients with high-titre antibodies against the GOR47–1 epitope, had no antibodies against the GOR1–125 epitope. We therefore conclude that there is no relationship between the GOR gene product and HCV infection in humans. We discuss here the possible mechanism of cross-reactivity between anti-HCV Ab and GOR47–1 epitope.

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MATERIALS AND METHODS

Isolation of the human GOR gene

We isolated the human GOR cDNA fragment as an estrogen-inducible gene from the human mononuclear cell line U937 using

RAP-PCR differential display technique (data not shown). Briefly, we extracted RNAs from U937 cells cultured with or without estrogen and synthesised cDNAs with degenerative 6-mer primer. The cDNAs were amplified by PCR using random primers and differential bands were discriminated by agarose gel electrophoresis [9]. One fragment which was overexpressed by estrogen was subcloned using the SURECLONE PCR cloning kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and determined its sequence with ABI PRISM DNA Sequencing kit (Perkin Elmer, Foster City, CA, USA) by ABI PRISM 377 DNA Sequencer (Perkin Elmer). A search of the DNA databank for homologous sequences, using the Genetyx-Mac computer program (Software Development Co., Japan), identified one fragment (accession number S78897) with a high homology to the chimpanzee GOR antigenic epitope [10]. Referring to the chimpanzee sequence, we determined the sequence of the entire protein coding region by PCR and translated it into the amino acid sequence of the human counterpart of the GOR gene product (Fig. 1a). Furthermore, we obtained GOR genomic fragments by PCR with GOR specific primers; sense primer was 5′CCCTCAAGAGACTGAAGGG3 and antisense primer was 5′CTTGTACTCAGAGGCCATGG3, from another 12 normal donors and 3 HCV patients. We determined the sequences of the critical region to exclude PCR error, mutation of transformed cell line, or polymorphism (Fig. 1b).

Construction of recombinant GOR1–125 protein

The partial GOR cDNA fragment corresponding to amino acids 1–125 (boxed sequence in Fig. 1), which does not overlap with the GOR47–1 epitope, was subcloned into the pGEX-6P-3 vector (Amersham Pharmacia Biotech) and production of the protein fused with glutathione-S-transferase (GST) as tag was induced by IPTG in BL21(DE3) cells. Cell lysate was purified with glutathione 4B sepharose and digested with ProScission protease (Amersham Pharmacia Biotech) at 5°C for 4 h. Remaining protease and GST fragments were absorbed by glutathione 4B sepharose. The volume and quality of recombinant protein were ensured by SDS-PAGE analysis (Fig. 2).

ELISA assays of antibodies against GOR47–1 epitope and GOR1–125 protein

Antibody against GOR47–1 epitope was detected with the NANB GOR EIA kit (Institute of Immunology, Tokyo) using the protocol recommended by the manufacturer. Antibody against the GOR1–125 epitope was detected by the direct sandwich ELISA procedure. In brief, 0·5 μg/ml GOR1–125 protein was coated onto a 96-well ELISA plate (Corning Costar, Cambridge, MA, USA) at 4°C overnight. After washing, diluted patient serum (1 : 20) was added and specific binding was detected by HRP-labelled goat anti-human IgG PAb (American Qualtex, San Clemente, CA, USA) and 2·2′-azido-di[3-ethyl-benzthiazoline sulphonate (6)] (ABTS) (Kirkegaard and Perry lab. Inc., Gaithersburg, MD, USA).

Patients

All patients were monitored at the Tokyo Medical and Dental university Hospital. Nineteen HCV patients were outpatients and were confirmed to be HCV carriers by detection of second or third generation antibodies against HCV and HCV RNA. The sera of non-HCV patients represented pooled sera of patients admitted to the First Department of Internal Medicine over several years, many with autoimmune diseases. All samples were obtained after obtaining informed consent.

Western blotting with patient sera

Western blotting of serum samples was performed using a standard protocol. Briefly, 10 μg recombinant protein were applied on SDS-PAGE and transferred onto a PVDF membrane. The membrane was blotted with a 1 : 200 dilution of patient serum. Specific signal was detected with HRP-labelled anti human IgG PAb (American Qualtex) and ECL reagent (Amersham Pharmacia Biotech).

Transient expression of GOR-green fluorescence protein (GFP) fusion protein in mammalian cells

The full-length GOR cDNA fragment, according to the sequence of human and chimpanzee cDNAs, was subcloned into the pEGFP-C1 vector (Clontech laboratories, Palo Alto, CA, USA) and transfected into COS7 cells using FuGENE6 reagent (Boehringer Mannheim Co., Indianapolis, IN) to identify intracellular localization. Transfectants were examined 48 h later by a fluorescence microscope (model BX50, Olympus Optical Co., Tokyo) equipped with WU filter.

RESULTS

Amino acid sequence of human GOR gene product; comparison with chimpanzee

The amino acid translation of the coding region of the GOR gene product is shown in Fig. 1a. Homology between the human and chimpanzee GOR genes was as high as 96% at the DNA level, and 94% at amino acid level. However, codon 319 of the chimpanzee sequence (TGG; Tryptophan) was replaced by TAG (a stop codon) in the human sequence. Since the region corresponding to the GOR47–1 epitope is located downstream of this codon, it is clear that the epitope cannot be translated and that the GOR gene product may be expressed as a truncated form in humans. We sequenced another 15 genomic clones derived from 12 normal donors and 3 HCV patients and confirmed this replacement in all samples (Fig. 1b). These results excluded replacement due to a PCR error, mutation in transformed cell lines, or polymorphism such as single nucleotide polymorphisms (SNPs). Subsequent to our studies, another group of investigators submitted long sequences including this region as a fragment of chromosome 8 (GENBANK accession No. AC012191) which confirmed this substitution.

Serum antibodies against GOR1–125 and GOR47–1 epitopes

We examined anti-GOR1–125 Ab titres in 201 serum samples. The mean titre in non-HCV subjects was similar to that of HCV patients (Fig. 3a). We next selected 41 samples of non-HCV patients with absorbance higher than 0·6. We examined background contributions in these samples by detecting reactions to wells without protein coating and recognized some samples with high titres of specific Ab against the GOR1–125 epitope (data not shown). Furthermore, reactivities to the GOR47–1 epitope were very low in 41 selected non-HCV patients whereas 12/18 HCV patients exhibited relatively high reactivities (Fig. 3b). No correlation was detected with autoimmune diseases in any patients (data not shown). These results suggest that reactivity to GOR1–125 may be distinct from that to the GOR47–1 epitope.

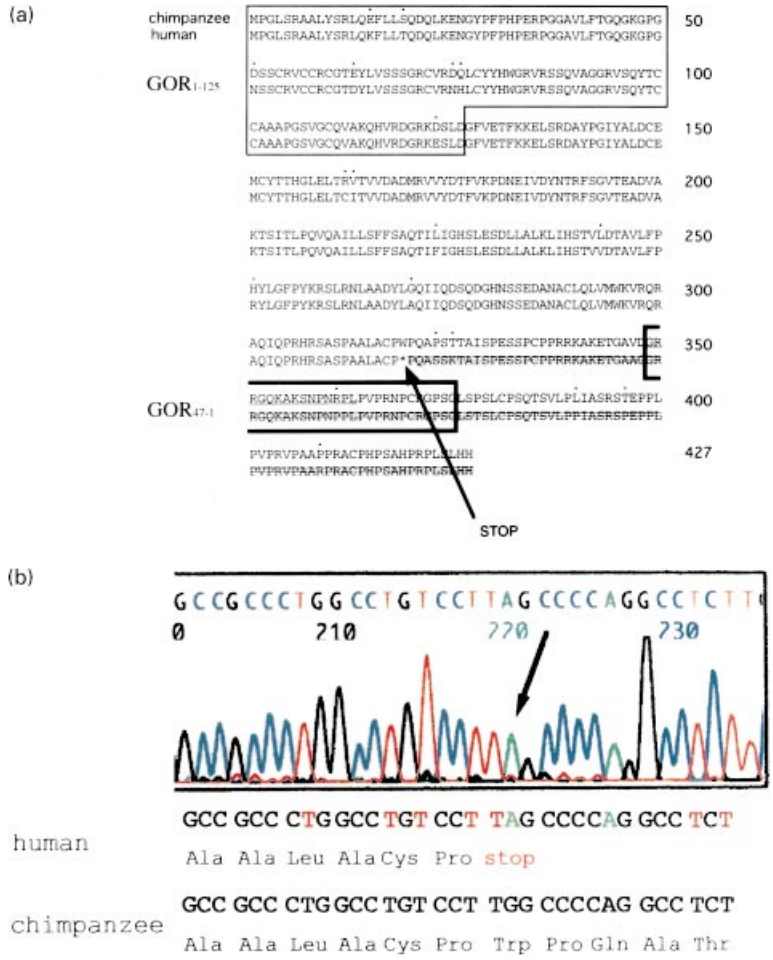


Fig. 1. Amino acid sequences of human and chimpanzee GOR gene translate (a) and representative base sequence including critical stop codon in human (b). Letters with dots at top represent non-identical sequences between the two species. The boxed sequence in N terminus was used for recombinant GOR1–125 epitope and the bold boxed chimpanzee sequence is the GOR47–1 epitope. Underlined sequence is suspected to cross-react to HCV core protein. Black arrow in (b) shows the critical single base replacement and it was identical in all samples analysed in this study.

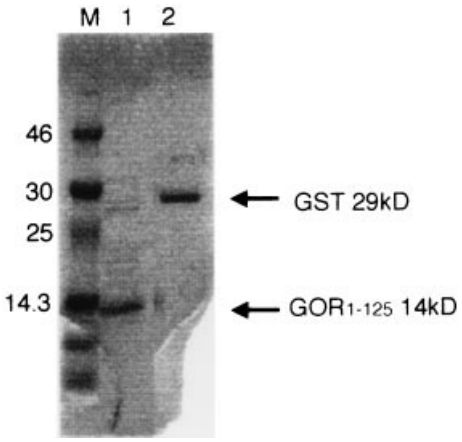


Fig. 2. The recombinant GOR1–125 protein in 15% SDS-PAGE stained with CBB. GOR1–125 protein is produced by *Escherichia coli* as fusion protein with GST and cleaved by site-specific protease (*see* Materials and Methods). GST and protease were removed using glutathione 4B sepharose. Lane 1, purified product; size 14 kD, lane 2, 29 kD purified GST as molecular size standard, lane M, molecular size marker. Numbers on the left show molecular sizes in kD.

Western blotting using sera of representative patients

To examine the specificity of the results of ELISA, we performed Western blotting against recombinant protein using patient sera and compared the results with those obtained by ELISA. As shown in Fig. 4, serum from MM reacted to recombinant GOR1–125 protein and revealed a corresponding band. In contrast, serum from KS, which had strong reactivity but high background by ELISA, did not exhibit any clear band of corresponding molecular size but several nonspecific bands including GST. Serum from MH exhibited a very low titre by ELISA and did not react to recombinant protein or background. These results were compatible with those of ELISA and confirmed that some patients expressed antibodies against the GOR1–125 epitope, which were independent of HCV infection.

Lack of correlation between anti-GOR Ab expression and serum IgG levels in HCV patients

None of the 18 HCV patients had autoimmune disorders. However, because serum IgG levels were examined in 12 of the 18 patients, we compared them with reactions to GOR epitopes (Fig. 5). There was no correlation between reactivity to GOR47–1

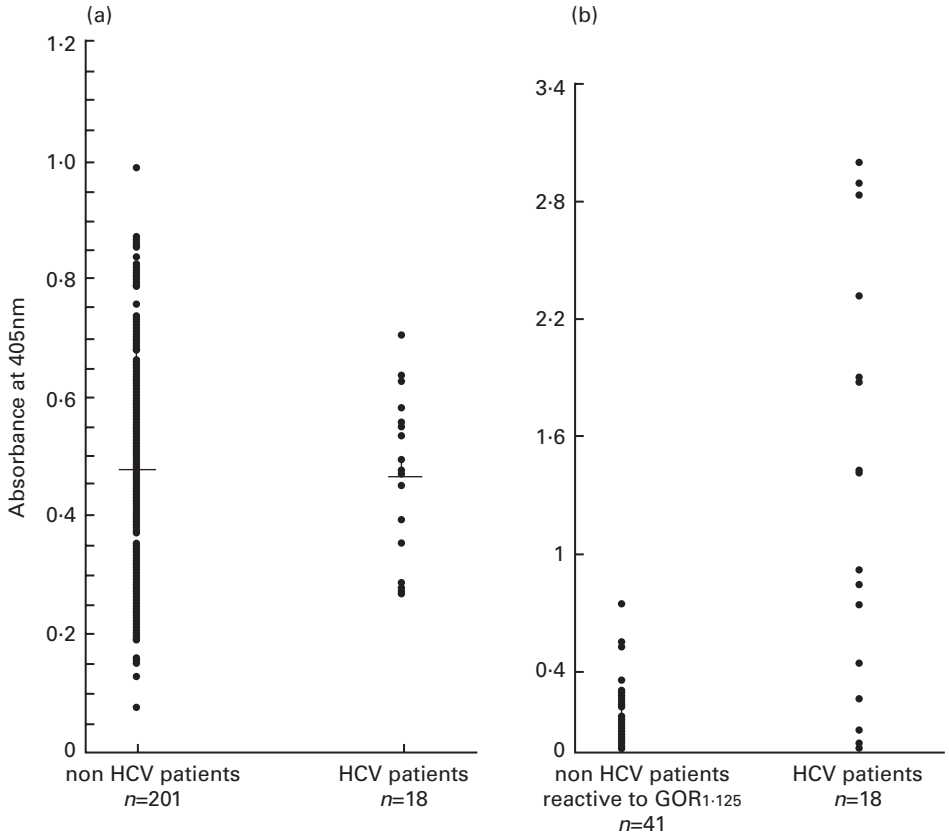
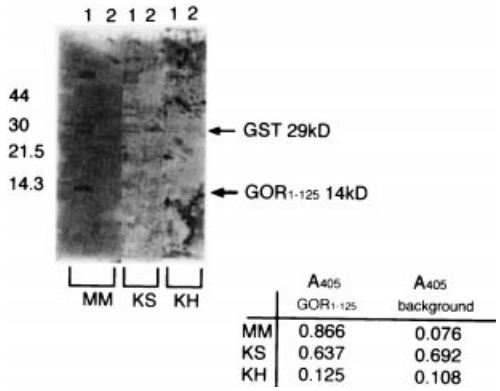


Fig. 3. Antibodies against GOR1–125 or GOR47–1 epitope in HCV and non-HCV patients. (a) Reaction of sera of patients with HCV and non-HCV against GOR1–125 epitope. Horizontal bar represents the mean value. (b) Comparison of reactivity to the GOR47–1 epitope between non-HCV and HCV. Note the low reactivity in 41 selected non-HCV patients whereas 12 of the 18 HCV patients exhibited a high reactivity.

or GOR1–125 epitopes and IgG levels, however, the background reactivity showed such correlation. Thus, it is possible that serum IgG level did not impair the reactivity to the GOR47–1 or 1–125 epitopes but might influence the background reactivity.



	A_{405} GOR1-125	A_{405} background
MM	0.866	0.076
KS	0.637	0.692
KH	0.125	0.108

Fig. 4. Western blotting of recombinant GOR1–125 protein using serum samples from representative patients. The results are compared with those of ELISA. Recombinant proteins were applied on SDS-PAGE, transferred to PVDF membrane, and blotted with diluted serum samples from each patient (MM, KS, or KH). Reactivities of these three patients to plastic wells uncoated or coated with GOR1–125 protein detected by ELISA are shown on the right.

Pattern of intracellular expression of GOR gene product correlates with gene sequences of humans and chimpanzees

Recombinant GOR protein fused with GFP could be expressed stably and formed granules that were localised around the nucleus (Fig. 6). A proportion of these granules we noted inside the cell nuclei, suggesting that GOR might be a nuclear protein. These results are in agreement with those reported previously by Mishiro *et al*. [10]. The pattern of intracellular expression of GOR gene product and stability correlated with the gene sequences of humans (Fig. 6a) and chimpanzees (Fig. 6b).

DISCUSSION

The anti-GOR47–1 peptide antibody detecting system is specific for HCV patients; it can detect over 70% of HCV carriers and is more sensitive than the first generation antibody, c100–3 [2]. It was interesting to note that the antigenic GOR47–1 peptide was derived from cDNA of the host animal rather than from HCV. The GOR47–1 peptide is therefore a self-peptide, cross-reactive with anti-HCV Ab, which may therefore induce autoimmunity through the mechanism of molecular mimicry. Based on this hypothesis, many workers have examined and discussed the relationship between GOR47–1 epitope and autoimmunity since various autoimmune phenomena can complicate HCV infection [4]. However, the issue has been controversial as there are reports both in support [1,8,11] and against [12,13] a relationship between

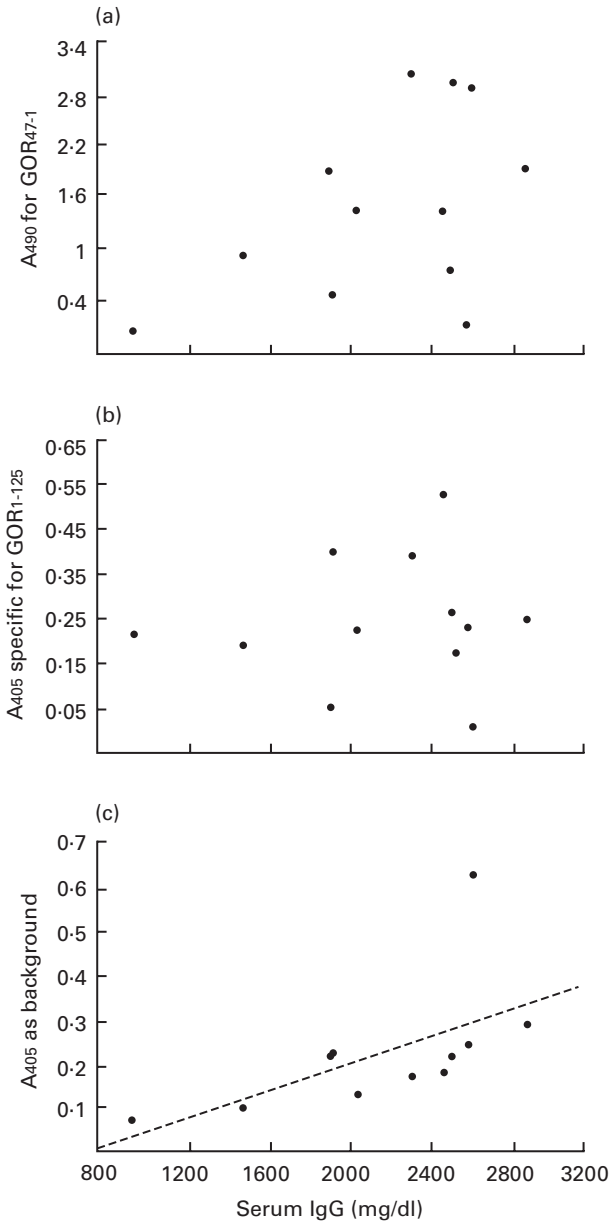


Fig. 5. Correlation between reactivity against (a) GOR47–1 epitope, (b) GOR1–125 epitope, or (c) noncoated wells and serum IgG level in HCV patients. The ordinate represents reactivity against GOR epitope or background while the abscissa represents the mean serum IgG levels of 12 HCV patients.

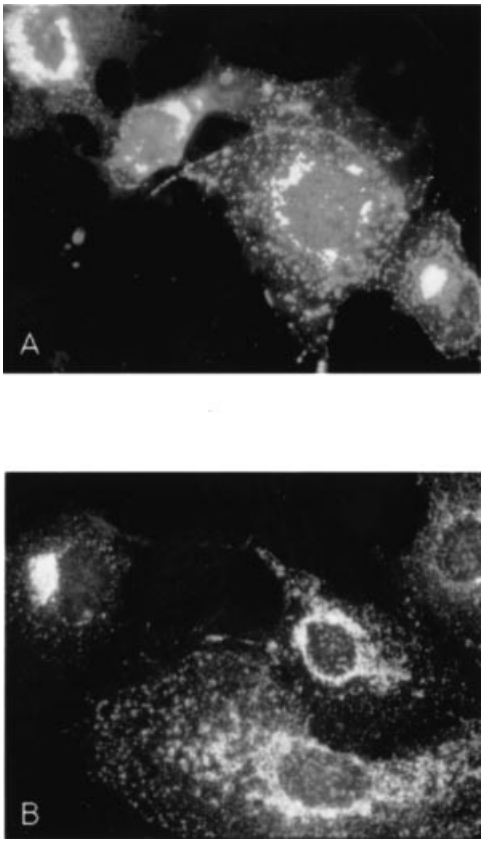


Fig. 6. Fluorescence microscopy of COS7 cells expressing GOR-GFP fusion protein according to human (A) and chimpanzee (B) gene sequence. Cells were observed 48 h after transfection using fluorescence microscope equipped with WU filter. Original magnification, × 400.

GOR and autoimmunity. Congia *et al*. [14] showed that the presence of anti-GOR47–1 antibodies without demonstration of HCV RNA was rare, suggesting that antibodies which react to the GOR47–1 epitope are not directed against the host-derived GOR gene product but rather against HCV. Zhang *et al*. [15] pointed out that 29 sera initially reactive to GOR47–1 epitope were also reactive for synthetic HCV core peptide. Moreover, no significant correlation has been found between expression of the anti-GOR47–1 Ab and autoimmune skin diseases such as porphyria cutanea tarda [16] and lichen planus [17].

In the present study, we demonstrated that the GOR47–1 epitope could not be translated due to a single base replacement at codon 319, and we confirmed this replacement in several genomic clones derived from normal humans or HCV-infected peripheral blood. Furthermore, another group of investigators recently submitted long sequences, encompassing the human GOR gene, to GENEBANK as part of the sequence of chromosome 8, which confirmed the presence of the stop codon. Taken together with our findings, we conclude that the GOR gene product is not related to HCV and HCV-related autoimmunity in humans.

The question then arises as to why the GOR gene or GOR47–1 epitope was detected in HCV-infected host animals? Three mechanisms may explain this phenomenon. Firstly, it is just a coincidence of antigenicity of the ubiquitous GOR gene, which can cross-react with HCV. Zhang *et al*. [15] analysed in detail the amino acid residues of the GOR47–1 epitope, which reacts with antibodies, and identified four essential residues, which are all conserved in HCV core protein. They reported that the relative antibody avidity of antibodies reactive to GOR was high for the HCV core peptide in most patients. Therefore, in most cases, anti-GOR Abs may be directed against HCV core protein. However, this does not fully explain why GOR cDNA was extracted from the cDNA library. Secondly, there may be some interspecies differences with respect to reactivity to HCV and progression of hepatitis between human and chimpanzee, despite their close genetic relationship. In chimpanzees, in which the GOR47–1 epitope is translated, it is possible that cross-reactivity of HCV and the GOR gene product might play a role in progression of

hepatitis and autoimmune phenomena related to HCV infection. To our knowledge, however, there are no studies to date that have investigated the relationship between anti-GOR47–1 Ab and HCV-associated autoimmunity in a chimpanzee disease model. Thirdly, following HCV infection, the GOR gene product may be induced with progression or regulation of hepatitis. Mishiro *et al*. [10] pointed out a nuclear targeting sequence and proline-rich residues in the GOR gene product. They also demonstrated nuclear localization of GOR gene product using hepatoma cells and serum of patients [10]. With respect to the truncated form of the human GOR gene product, we observed stable expression and nuclear localization using a recombinant fusion protein with GFP (Fig. 6). It is therefore possible that the GOR gene product is a nuclear protein that contributes to the development or regulation of hepatitis. Furthermore, we (unpublished observation) and Dennin *et al*. [18] found a conserved homologous gene in other animals and plants, suggesting that the GOR gene might be universally important and has basic physiological roles. Our ongoing studies include analysis of the physiological roles of the GOR gene product in an attempt to fully elucidate the relationship between the gene and HCV.

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