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主論文

Integration of woodchuck hepatitis virus (WHV) DNA at two chromosomal sites (V_K and *gag*-like) in a hepatocellular carcinoma

(Nucleotide sequence; viral-chromosome junction; immunoglobulin V_K region; homologous recombination; *X*-cellular gene fusion)

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Abbreviations; aa, amino acid(s); bp, base pair(s); *c*, gene encoding Core antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IgV_K, immunoglobulin L-chain V_K; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; PCR, polymerase chain reaction; *preS*, gene encoding PreS protein; *s*, gene encoding Surface antigen; WHV, woodchuck hepatitis virus; *x*, gene encoding X protein.

SUMMARY

Integration of woodchuck hepatitis virus (WHV) DNA into the liver DNA of a woodchuck infected by the virus was investigated. Clonal viral integration was not detected 3 months before the appearance of four hepatocellular carcinomas (HCC). Integration of the viral DNA was detected in all four HCCs, of which one was chosen to determine the structure of the viral integration completely in a single tumor. The integration occurred in two sites. One part contains the viral DNA from the middle of S to two-thirds of the way through the X gene with no structural changes. The coding frame of the truncated X gene continues into the chromosomal sequence to make a possible fusion protein. The integration seems to have occurred by recombination within two direct repeats of the viral genome in one junction and by homologous recombination between viral DNA and chromosomal DNA in the other junction. The viral DNA is integrated into a spacer of the immunoglobulin L-chain V_K (Ig V_K) region without any chromosomal rearrangement accompanying the integration. The viral DNA at the second site has a complex structural rearrangement: a part of the preS gene is duplicated and attached to the terminus of the C gene in a head-to-tail fashion, followed by three small fragments derived from other parts of the viral DNA. The integrated preS gene has its own 5' regulatory element and a coding frame consisting of the truncated preS gene, the other parts of viral DNA and the chromosomal sequence. At both viral-chromosome junctions twenty-eight nt of the chromosomal DNA are duplicated as often seen in retrovirus integration. At the viral-viral junctions, viral DNA may have been rearranged by possible intramolecular homologous recombination using hexamers within the viral genome. The viral DNA is integrated in the vicinity of a retroviral gag-like open reading frame associated with highly repetitive sequences.

INTRODUCTION

Integration of human hepatitis B virus (HBV) has been studied to examine a direct correlation between HBV infection and hepatocarcinogenesis. Woodchuck and woodchuck hepatitis virus (WHV) is a good model system because the hepatocellular carcinomas (HCC) occur relatively in short time after the infection by the virus and therefore there would be more chance to observe a direct relation between the viral infection and hepatocarcinogenesis. In some cases of integration of both HBV in human and WHV in woodchuck liver, insertional activation of genes involved in cell proliferation was observed as the result of viral integration (Dejean et al., 1986; Hsu et al., 1988; Fourel et al., 1990; Wang et al., 1990). In particular, Fourel et al. reported that six cases out of 30 WHV-related HCC have insertions of WHV DNA adjacent to cellular N-myc.

Besides the insertional activation of cellular genes, there remains a possibility that products of the integrated viral gene may stimulate cell proliferation through activation of important cellular functions in trans. The viral X protein has been shown to stimulate transcription from many viral and cellular gene promoters (Twu and Schloemer, 1987; Spandau and Lee, 1988; Aufiero and Schneider, 1990; Levrero et al, 1990). Although X coding region is truncated near its 3'end as the result of integration, such a truncated X protein is reported to retain transactivating activity (Wollersheim et al., 1988; Takada and Koike, 1990). Koshy and his colleagues reported recently that a transcriptional transactivator function not present originally in the intact gene was generated by the truncation of the 3'-portion of the HBV preS/S sequences during integration (Caselmann et al., 1990; Kekulé et al., 1990).

It seems that integration of the viral DNA is often directly involved in

carcinogenesis by interaction with cellular oncogenes either in *cis* by providing enhancer-promoter sequences or in *trans* by producing transactivating factors. It is therefore important to analyze more cases of integration in order to identify new cellular oncogenes as well as detecting hitherto unknown functions of the viral gene products. Here we report a case of woodchuck HCC in which WHV DNA is integrated into two sites, one into an immunoglobulin V_K encoding region and the other into a gene homologous to retrovirus *gag* gene.

RESULTS AND DISCUSSION

(a) Integration of WHV DNA into the liver DNA of a woodchuck infected with WHV.

A woodchuck infected chronically with WHV and therefore containing WHV surface antigen in the serum was bred under the careful observation by ultrasonography until it carried tumors in the liver. The animal was sacrificed and the whole liver was taken by an abdominal operation. Four multicentric HCC, T1 to T4, were found in separate regions of the liver. DNA samples were isolated from these liver samples including the one cut out from an apparently normal region. Clonal integration of the viral DNA was not observed in liver samples isolated three months before the appearance of the tumor or from the normal region of the tumor bearing liver. In contrast, clear integration in more than one site was detected in all four tumors (Fig. 1A). For the further structural analysis, we chose one tumor T1 in which integration into two separate sites were clearly shown by the Southern blot hybridization (Fig. 1B and C). Fig. 2 shows a whole picture of the structure of viral and chromosomal DNA at the two integration sites deduced

from the cloning and sequence determination as described below.

Fig. 1B shows that two BamHI fragments, B7 (7.8 kb) and B3 (3.0 kb), and two EcoRI fragments, 3.0 and 2.6 kb, contain viral DNA. Each band was shown to contain different portion of WHV genome by the Southern blot hybridization using different portions of WHV genome as the probes (Fig. 1B). Thus, the region from gene X to C was located in B7 and EcoRI 2.6 kb fragments, but not in B3 and EcoRI 3.0 kb.

(b) Structure of the integrated viral DNA and of the viral-chromosome junction at B7

The B7 fragment containing the viral DNA was cloned from the tumor T1 using WHV DNA as a probe. After the determination of chromosomal DNA sequence near the viral DNA, the normal counterpart of the B7 was cloned from the normal region of the same liver using the unique chromosomal sequence as a probe (Fig. 2A). In B7, the viral DNA, 1852 bp in length, corresponding to nt 593 to 1844 of WHV2 genome (Kodama et al., 1985) was integrated without any rearrangement, but contains 2.8% base substitutions as compared with the WHV2 sequence (Fig. 3A). The viral sequence in B7 is nearly a quarter of the viral genome, a region from the middle of gene S to the 5'-two thirds of gene X. The region contains no intact viral genes, but contains the transcriptional enhancer and the promoter for gene X. The coding frame of the truncated X continues into the chromosomal sequence to make a possible fusion protein "Xc", 119 aa from X gene and 37 aa from the chromosomal sequence. In addition, there is a putative signal for poly(A) addition (TATAAA) about 90 bp downstream from the fused ORF. Production of the fused protein and its possible function in relation to the cell growth are remained to be examined.

One junction to the chromosome occurs within the gene X and is located between the two direct repeats, DR1 and DR2. Fifteen nt of the viral DNA are

repeated twice at the 3' junction to the chromosome. Since the formation of the junction at or near the direct repeats was observed most often in many HBV integration so far analyzed (Nagaya et al., 1987; Zhou et al., 1988), the junction in gene X as well as formation of the repeat of the 15 nt may be the product of the primary recombination of the viral genome to the chromosome. The terminal three nt, GGT, at the 5' junction were found both in WHV DNA and normal chromosomal DNA (Fig. 4), suggesting that the homologous recombination occurred at this site. Similar observation of the presence of two or three nt common to viral and chromosomal DNA at the junction have been reported (Berger and Shaul, 1987; Shih et al., 1987).

(c) Immunoglobulin V_k encoding gene is the site of the integration in B7.

To determine the site of the integration of the viral DNA, regions flanking the viral DNA in B7 were sequenced and searched for homologous sequences using the Genetic Sequence Data Bank (GenBank). In B7, two regions, on each side of the viral DNA, were found to be significantly homologous with mouse immunoglobulin V_k (Fig. 2A and 5) (Heinrich et al., 1984). In both cases the V_k-coding sequences are interrupted by multiple numbers of stop codons, indicating that they are pseudogenes. In addition to the pseudogenes there are many sequences characteristic of Ig V_k gene: a combination of deca- and pentadeca-nt with TATA box (a control unit for transcription of Ig gene, Falkner et al., 1984), an exon for leader peptide, and a combination of hepta- and nona-nt (a signal for V-J joining, Max et al., 1979; Sakano et al., 1979) (Fig. 5). The B7 counterpart from the normal liver shows restriction enzyme site map identical to that of the chromosomal region of B7 (Fig. 2A). This indicate that there occurs no rearrangement in the chromosome during integration.

(d) Structure of the integrated viral DNA and of the viral chromosome

junction at B3.

The clone B3 contained only one viral-chromosome junction and the other end was terminated at the BamHI site within the WHV genome. In order to know location of the other viral-chromosome junction and the structure of the flanking chromosome, we performed Southern blot analysis of DNA from T1 and normal part of the same liver. A unique fragment of chromosomal sequence within B3 (probe c in Fig. 2A) and WHV subgenomic fragment downstream from BamHI site (probe d in Fig. 2B) (nt position 1 to 700) were used as probes. Comparison of the restriction enzyme cleavage sites between the two kinds of DNA revealed that the whole length of the integrated virus at B3 should be about 1.3 kb and that there seems to be no discernible rearrangement of chromosomal DNA accompanying the viral integration (data not shown). By unknown reason, attempts to clone the fragment flanking the B3 was unsuccessful. Therefore a 4.2 kb EcoRI fragment representing this region of normal allele was first cloned from genomic library of a woodchuck hepatocyte cell line WLC3 (Lee et al., 1987) using the same chromosomal DNA probe (probe c in Fig. 2A) and nt sequence of the region corresponding to viral-chromosome junction was determined (see Fig. 2A). Polymerase chain reaction (PCR) was then performed against T1 genomic DNA using oligos within the WHV sequence and host DNA sequences as primers (PW and CW in Fig. 3B). Then we determined the structure of the remaining portion of the integrated viral DNA and the second viral-chromosome junction by sequencing the amplified PCR product.

The nt sequence of the viral DNA shows complicated rearrangement of the viral genome (Fig. 3B). A part of the preS region is duplicated and connected to the C-terminal end of gene C, followed by the regulatory region of preS and three small parts of other regions of the viral DNA. The second preS is truncated at aa 167 and continues into the following viral and flanking

chromosomal sequence to make a possible fusion protein of 246 aa. There is a putative signal for poly(A) addition about 340 bp downstream from the fused preS coding region. Production and its possible function of this fused protein are also to be studied.

At both junctions twenty eight nt of the chromosomal DNA are duplicated as often seen in retrovirus integration and there existed three nt common to viral and chromosomal DNA like that was detected at the viral-chromosome junction in the clone B7. At the second and the third viral-viral junctions, viral DNA may have been rearranged by possible intramolecular homologous recombination using two sets of common hexamers within virus genome (nt position 156 to 1612 and 1017 to 1022, and nt position 1065 to 1070 and 1206 to 1211) (Fig. 6).

(e) Retroviral gag-like gene is found near the 3' viral-chromosome junction.

Chromosomal location of the integration site of the clone B3 is characterized by the repetitive sequence which are homologous to those identified as an Alu-like repetitive sequence common in rat and mouse (Fig. 2A) (Young et al., 1982; Yang-Yen et al., 1985). Since search for homologous DNA sequences failed to reveal any long stretch of significant homology with known sequences, we translated the DNA sequences of the clone B3N into amino acids in all possible reading frames. In the 3' end of the clone B3N, that is 1.7 kb downstream from the viral integration site, we found an ORF consisting of 351 aa. But the ORF is truncated at 5' end of the clone and also has -1 frame shifting in the middle, so that either a splicing event or a ribosomal frameshift is necessary for the expression of this ORF. Comparison with Protein Data Bank (NBRF) revealed that the ORF, except for 50 aa at the carboxyl(C)-terminus, has a significant homology with C-half region of retroviral Gag proteins and with the middle region of

Gag-protease polyprotein in intracisternal A particle or of IgE binding protein (Fig. 7). However the ORF is followed by Alu-like repetitive sequence instead of protease, polymerase and envelope homologous sequences. In C-half region of the ORF, there are two zinc finger motifs and incomplete stretches of direct repeats suggesting that this ORF encodes a protein belongs to a family of transcriptional factors.

(f) Conclusions

We examined the structure and site of the integration of WHV DNA in a single HCC. Viral genome was integrated in two separate sites, B7 and B3.

(1) At B7, the viral genome from the middle of S gene to the 2/3 of X gene was integrated into a spacer of Ig V_K region. The frame of 3' truncated viral X gene continued into chromosomal sequence to make a possible fused protein which may act as a transcriptional transactivator.

(2) At B3, the viral genome from 5 separate portions was arranged in one orientation and integrated in the vicinity of retroviral gag-like ORF. The viral integration may affect the expression of the gag-like gene. The integrated viral genome contained an ORF consisting of 3' truncated preS gene followed by viral and chromosomal sequences. Such a truncated and fused preS gene may be expressed and have a transactivating activity.

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Legend to Figures

Fig. 1. Integration of WHV DNA in various liver DNA samples from a WHV-infected woodchuck.

Liver samples were obtained by biopsy 3 months before HCC development (Pre), or from four separate tumors (T1-T4) and normal part of the same liver after abdominal operation (N). DNA was isolated and subjected to electrophoresis in 0.8 % agarose gel with or without digestion by restriction enzymes, Eco, EcoRI; Bam, BamHI. DNA fragments containing the viral DNA were detected by Southern blot hybridization using a whole or a part of WHV genome (see D-a and D-b) as probes labeled by [α-^{32}P] dCTP (Amersham International Ltd., England). (A) BamHI digested DNAs from various samples were hybridized with a whole WHV genome. (B) T1 DNA probed by the whole viral DNA. (C) T1 DNA probed by X region of the viral DNA, probe b in (D). Arrows indicate fragments integrated with viral DNA.

Fig. 2. Schematic representation of a whole picture of two integrations.

(A) Restriction site map of clones, B7 and B3, and B7N and B3N (clones from normal counter part of B7 and B3, respectively). The viral DNA (shadowed arrow boxes) was shown on the chromosomal fragments (thin lines). Sequenced area are thickly lined. Dotted lines indicate the regions estimated by Southern hybridization but not cloned yet. Locations of Ig V_K pseudogenes (dotted arrows) and a putative ORF for X-cellular fusion protein (arrow "Xc") on B7 are indicated below B7. Two thick lines (a and b) under B7 are fragments used to clone the normal chromosomal counterpart, B7N. Thin lines with two opposite arrows above B3 denote the location and direction of PCR primers and the amplified product. Below B3, the fragment used to clone B3N (line c) and a putative preS-cellular fusion protein (arrow "Sc") on B3 are

indicated. Below B3N, arrowheads indicate approximate location of Alu-like repeats on the chromosome B3N, and the open arrow box indicates the putative ORF shaded for gag homologous region. Small open triangle shows the location of frameshift. Restriction enzymes are: B, BamHI; Dr, DraI; E, EcoRI; H, HindIII; Hh, HhaI; Kp, KpnI; P, PstI; Pv, PvuII; S, SacI; X, XbaI. (B) Portions of viral DNA integrated in B7 and B3 are illustrated. ORFs on the viral genome are schematically shown by open arrows: C, WHcAg; P, pol gene; PreS, preS region; S, WHsAg; X, X gene. Locations of DR1 and DR2 (small triangles) and the enhancer (En) are shown. Thick line (d) under WHV shows the probe used in Southern hybridization. At the bottom, the viral DNAs detected by sequencing are shown by solid bars with the nucleotide position number at the junction.

Fig. 3. Nucleotide sequence of the integrated viral DNA and flanking cellular chromosomal DNA.

The viral nt sequences are boxed. The aa sequence of ORFC, ORFS, ORFX, and preS are shown, but that for pol was omitted to avoid complication. Start sites of ORFX and preS, ends of ORFS, ORFC and ORF of X-cellular and preS-cellular fusion protein, and the direct repeat 2 (DR2), are indicated. Black dots on the sequence indicate nt differing from those of WHV2 sequence (Kodama et al., 1985). Putative signal for polyA addition (TATAAA) is boxed. (A) Sequence in B7 fragment. Nt number 1 is the BamHI site of the B7 fragment. The enhancer-promoter region of X (En and Xp; Shaul et al., 1985; Treinin and Laub, 1987) and the 15nt repeat are underlined. A putative X-cellular fusion protein is shown by the aa sequence extending from the right junction. Amino acid sequences with parenthesis are derived from the chromosomal DNA. (B) Sequence in B3 fragment region. Nt number 1 is the

BamHI site of the B3 fragment. A part of preS is duplicated and fused to C near the end of the ORF, followed by the other three parts of the viral sequence. 5' regulatory element (TATA box) and transcription start site of preS gene are underlined. A putative preS-cellular fusion protein is shown by the aa sequences. Twenty eight nt duplicated at the both viral-chromosome junctions are shown by double underlines. Sequences overlined with arrows (PW and PC) show oligonucleotides used as primers in PCR experiment. The BamHI site used in the cloning of B3 is indicated at nt position 3003.

Fig. 4. Sequence of the viral-chromosome junctions in B7 and B3 region.
The nt sequence of viral-chromosome junctions in B7 and B3 region are shown together with those of corresponding regions of WHV2 (Kodama et al., 1985) and normal counterpart (B7N, B3N). Homologous bases are marked by asterisks. The extra 15 bp sequence repeated at the right junction in B7 is underlined. Twenty eight nt of the chromosomal DNA duplicated at the both junctions in B3 are shown with double underlines. Three nt common to viral and chromosomal sequence at the junctions are boxed.

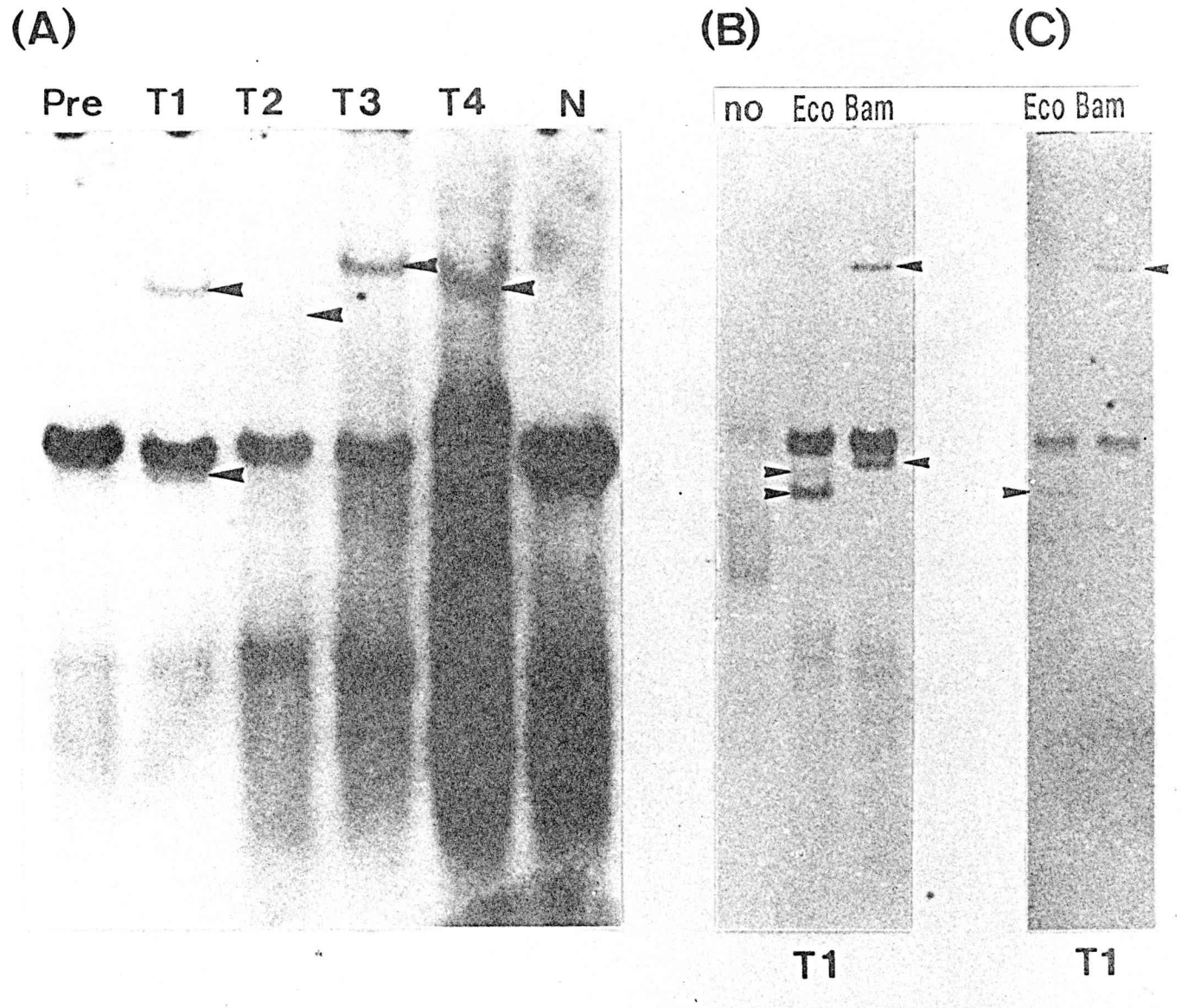
Fig. 5. Sequence of the Ig V_K region flanking the viral DNA integrated in B7.
Since direction of ORF of the V_K pseudogene is opposite to that of the viral sequence (see Fig. 2A), the sequence of the strand complementary to that in Fig. 3A is presented. From the 6th to 9th line, the sequence of mouse germline Ig V_K gene (V-kappa-21C, Heinrich et al., 1984) is also shown under that of B7. Asterisks indicate homologous nt. Slashes show the boundaries where the homology with B7 is lost. V_K pseudogene is underlined together with a putative leader sequence. Sequence similar to promoters for V_K gene (TATA box), pentadeca- (pd) and deca-nt (dc) are indicated. Sequences similar to V-J junction, hepta and nona sequences, are also shown.

Fig. 6. A model for viral DNA rearrangement mediated by intramolecular homologous recombination in B3.

The nt sequences with open and shadowed arrows denote each pair of common hexamers and their directions within WHV. Above and below the sequences, nt position corresponding to WHV2 are numbered. Oblique lines between two sequences are possible sites of recombination.

Fig. 7. Amino acid sequence homology of B3N ORF with some kinds of Gag protein and IgE-binding factor.

(A) Alignment of the putative amino acid and DNA sequence of the B3N ORF with the gag gene product of Mason-Pfizer monkey virus (MPMV) (Sonigo et al., 1986), mouse mammary tumor virus (MMTV) (Moore et al., 1987), simian acquired immune deficiency syndrome retrovirus (SRV1) (Power et al., 1986), mouse intracisternal A particle (IAPMO) (Mietz et al., 1987), hamster intracisternal A particle (IAPHA) (Ono et al., 1985) and with rat IgE-binding factor (IgEBF) (Martens et al., 1985). The beginning of B3N ORF is 3' terminal end of the clone B3N. Chemically similar amino acids are boxed. Gaps (-) are inserted to increase sequence similarities. Slashes show the boundaries where the homology with B3N ORF is lost. Two stretches of zinc finger motif are drawn with bold italic letters. Direct repeats are shown by thick lines with arrows over nt sequences. (B) Comparison of the genomic organization of B3N ORF with MPMV, MMTV, SRV1, IAPMO, IAPHA and IgEBF. Homologous region with B3N ORF is shaded. Three small triangles in IAPHA indicate terminal codons appeared within the frame. Abbreviations are: gag; gag ORF; prt, protease ORF; pol, polymerase ORF; env, envelope ORF.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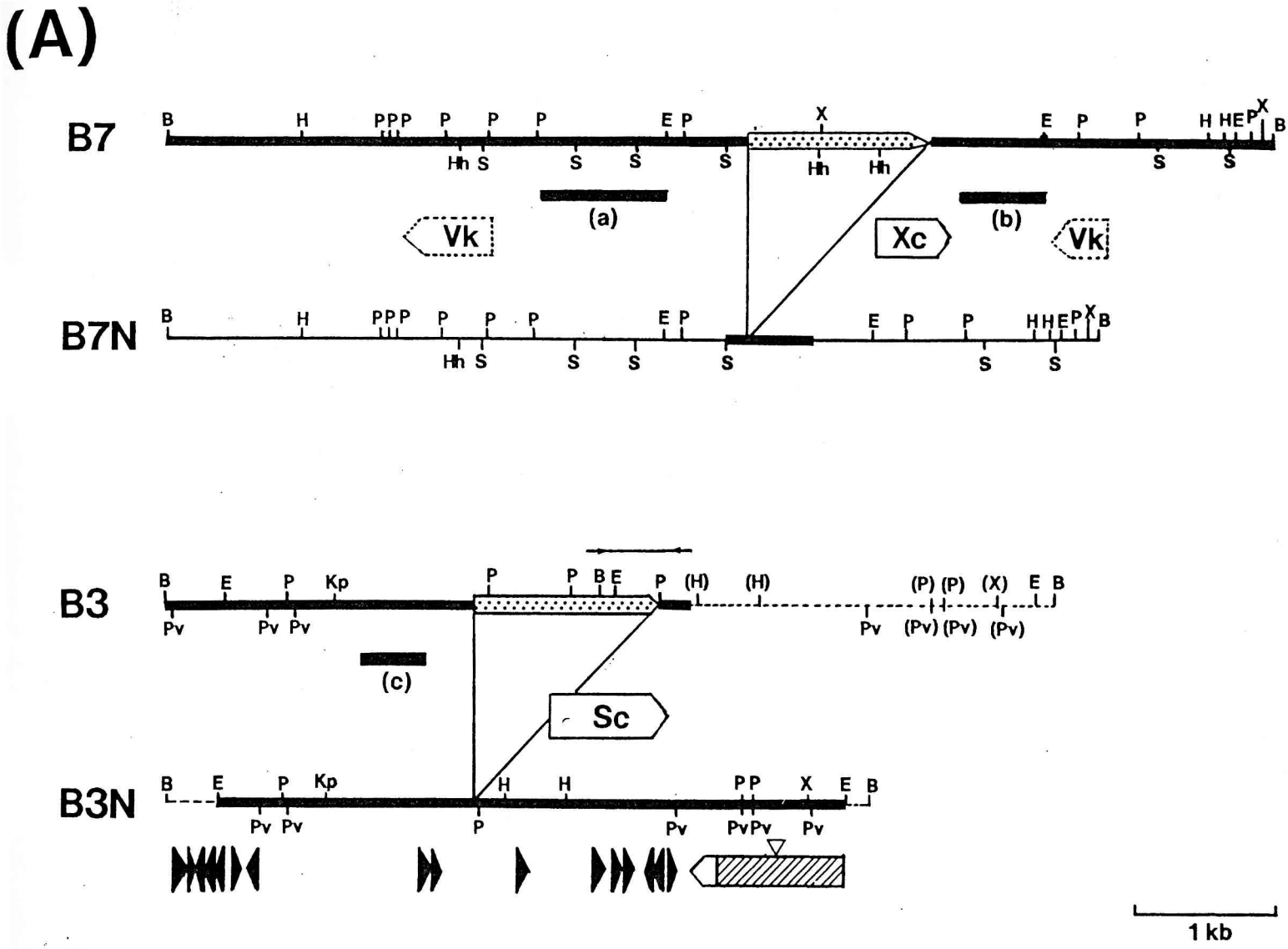
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S
En
X
C
PreS
WHV
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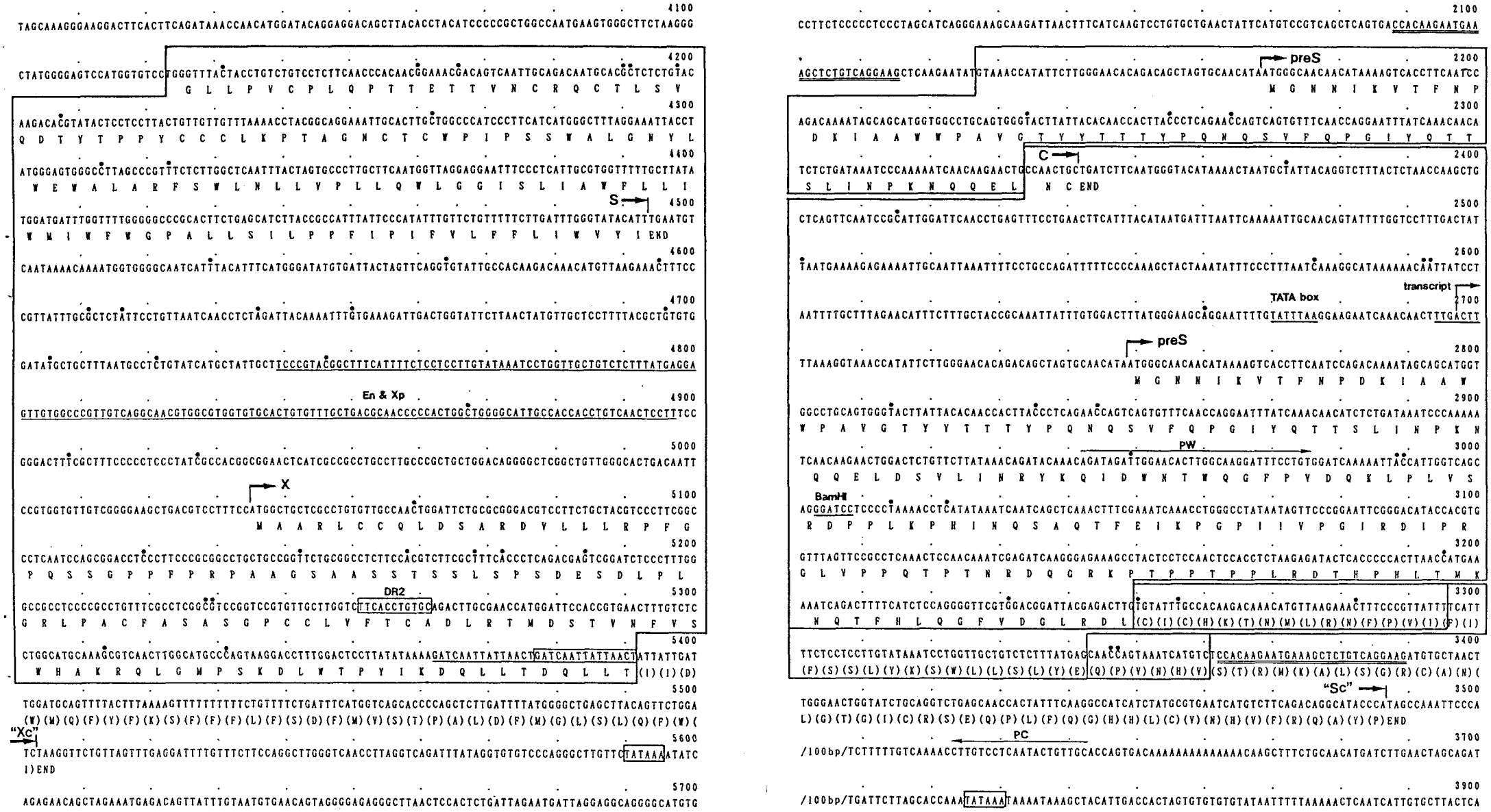
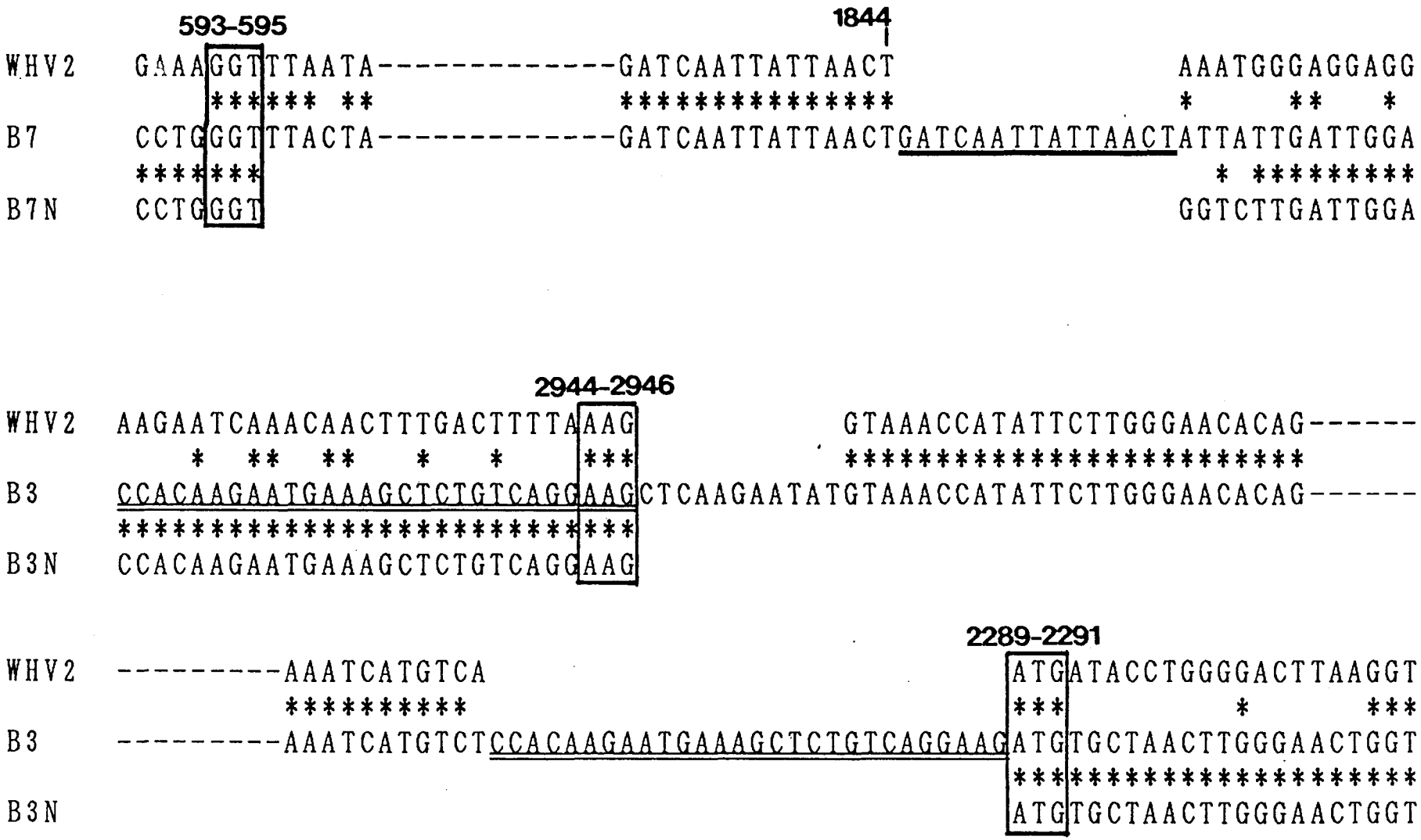

(A) B7
(B) B3
En & Xp
DR2
X
S
C
preS
PW
TATA box
transcript
BamHI
"Xc"
"Sc"
PC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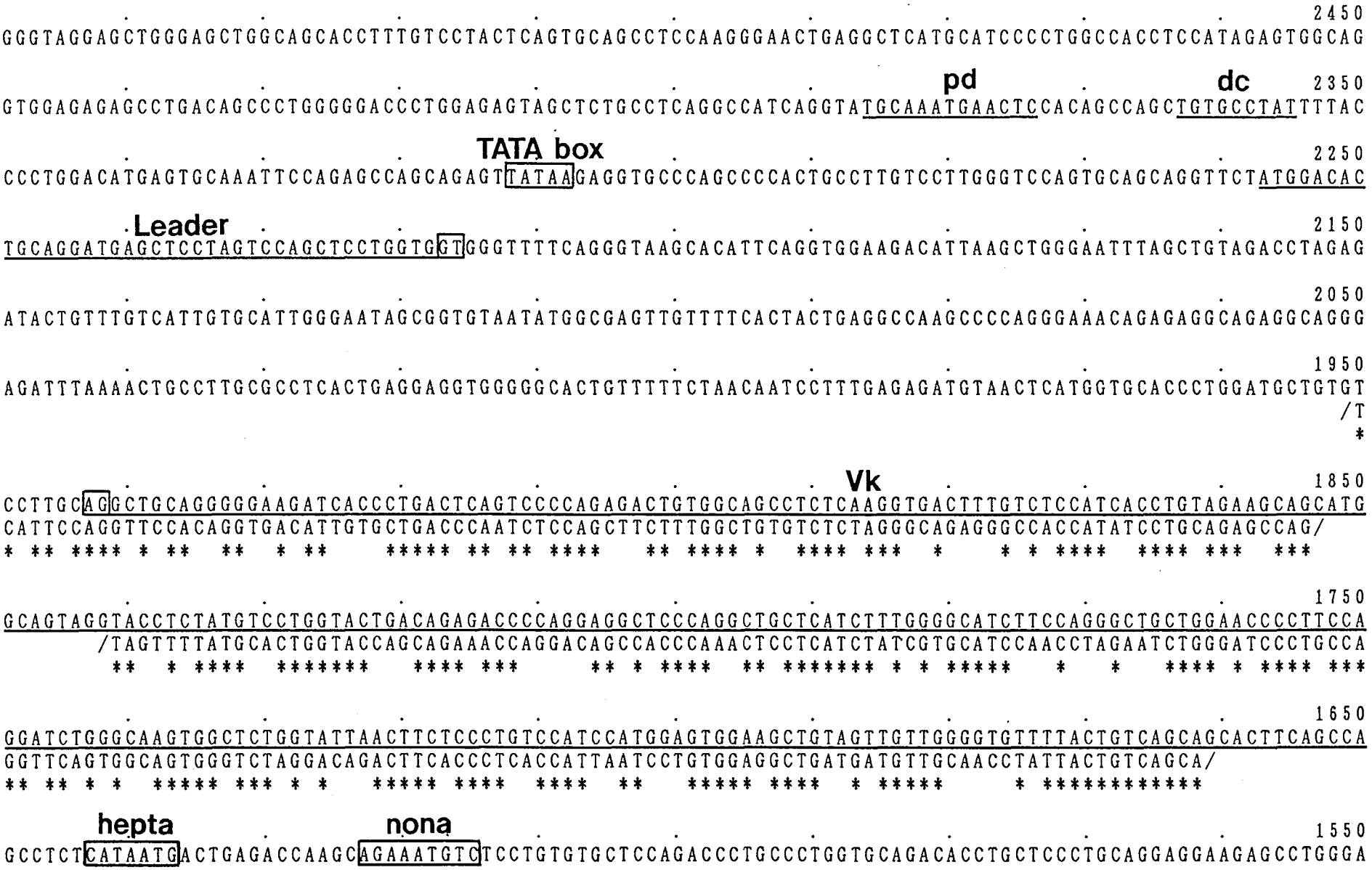
B7 CCTGGGTTTACTA----------GATCAATTATTAACTGATCAATTATTAACTATTATTGATTGGA
B7N CCTGGGT GGTCTTGATTGGA
2944-2946
WHV2 AAGAATCAAACAACTTTGACTTTTAAAG GTAAACCATATTCTTGGGAACACAG------
B3 CCACAAGAATGAAAGCTCTGTCAGGAAGCTCAAGAATATGTAAACCATATTCTTGGGAACACAG------
B3N CCACAAGAATGAAAGCTCTGTCAGGAAG
2289-2291
WHV2 ----------AAATCATGTCA ATGATACCTGGGGACTTAAGGT
B3 ----------AAATCATGTCTCCACAAGAATGAAAGCTCTGTCAGGAAGATGTGCTAACTTGGGAACTGGT
B3N ATGTGCTAACTTGGGAACTGGT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pd
dc
2350
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TATA box
2250
CCCTGGACATGAGTGCAAATTCCAGAGCCAGCAGAGTTATAAGAGGTGCCCAGCCCCACTGCCTTGTCCTTGGGTCCAGTGCAGCAGGTTCTATGGACAC
Leader
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1950
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/T
*
Vk
1850
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* ** **** * ** ** * ** ***** ** ** **** ** **** * **** *** * * * **** **** *** ***
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** * **** ******* **** *** ** * **** ** ******* * * ***** * * **** * **** ***
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** ** * * ***** *** * * ***** **** **** ** ***** **** * ***** * ***********
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nona
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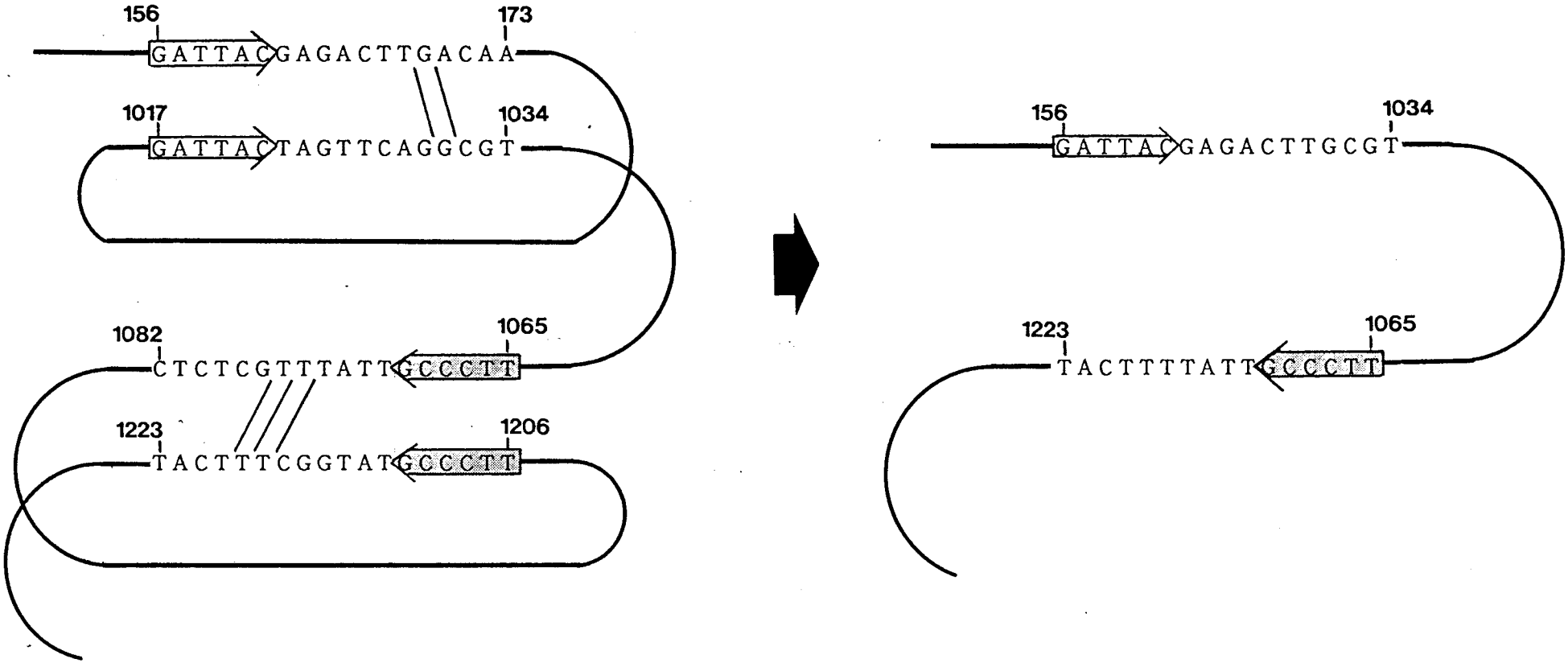
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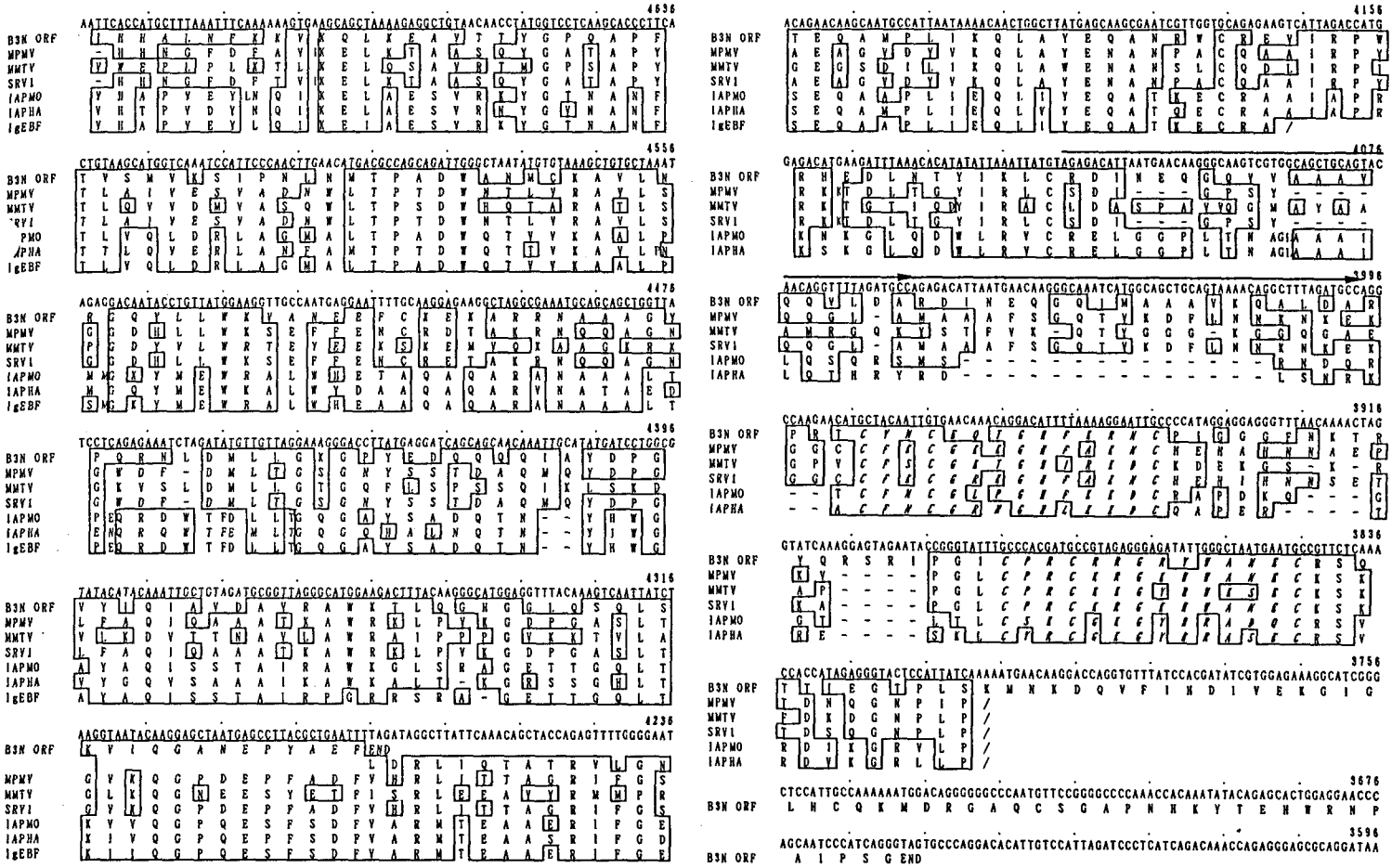
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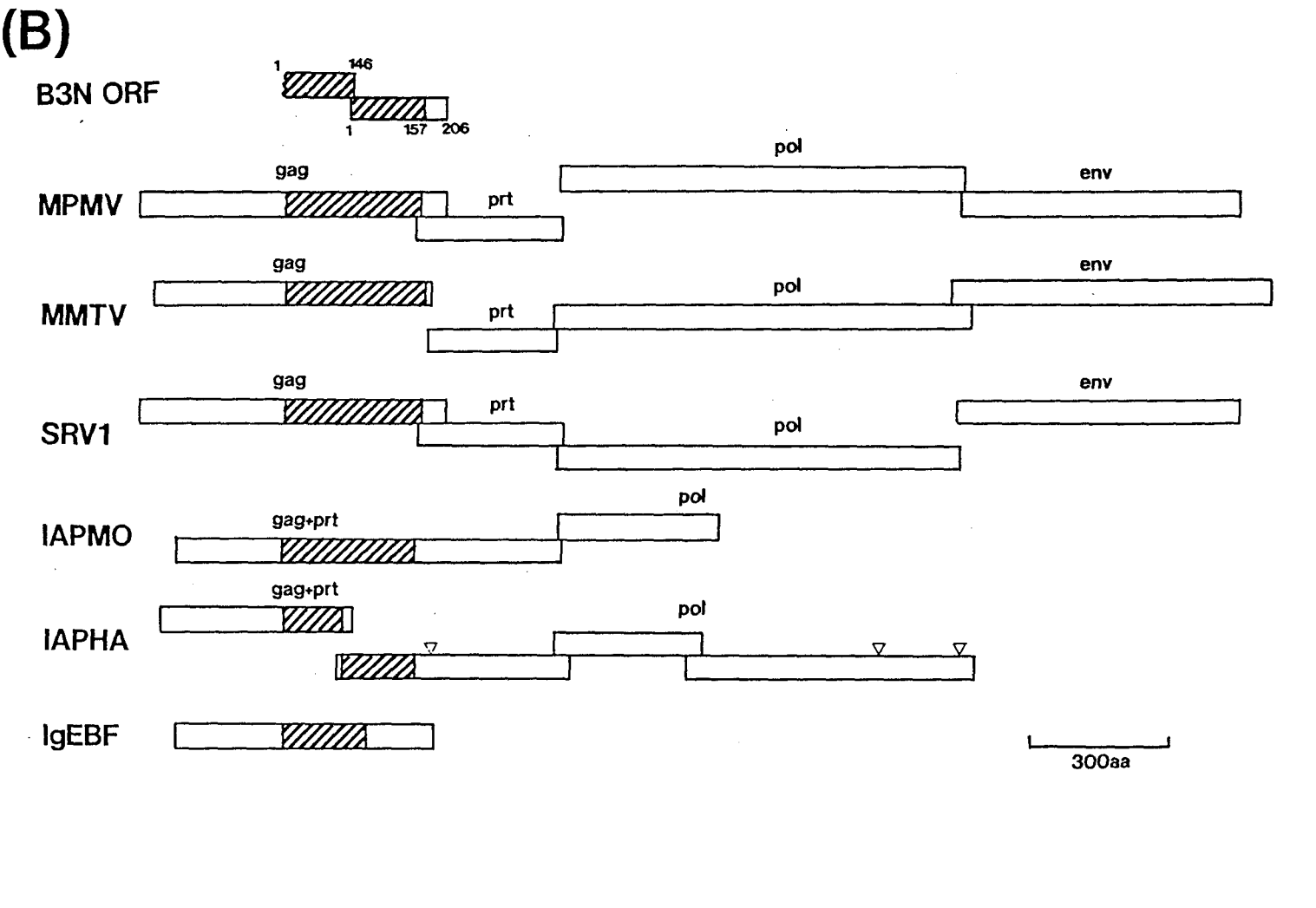
MPMV
MMTV
SRV1
IAPMO
IAPHA
IgEBF


(B)
B3N ORF
1
146
1
157
206
MPMV
gag
prt
pol
env
MMTV
gag
pol
prt
env
SRV1
gag
prt
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env
IAPMO
gag+prt
pol
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gag+prt
pol
IgEBF
300aa

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Synthesis of human gamma-glutamyl transpeptidase (GGT) during the fetal development of liver

(Recombinant DNA; immunostaining; messenger RNA; monoclonal antibody; hepatocarcinogenesis)

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SUMMARY

We have determined expression of human *GGT* gene encoding γ-glutamyl transpeptidase (GGT) during fetal development of liver using the Northern-blot analysis with a cloned human *GGT* cDNA and immunohistochemistry with a monoclonal antibody. *GGT* mRNA could be detected as early as the 12th week of gestation. It then increased gradually to a peak of approx. threefold the amount at week 12, at week 40, just before birth. The size of the mRNA in the fetal liver was 2.7 kb and mRNA of the same size was detected both in the human fetal kidney and human hepatocellular carcinoma as well as normal adult liver. Immunohistochemical analyses show that GGT increased as the fetal liver developed in parallel with the increase in mRNA. Histochemically, GGT was shown to be located in the wall of bile canaliculi when synthesis was low in early development, but to be distributed, in addition, all over the cell membrane of the fetal hepatocytes when synthesis was high at the later stage of development.

INTRODUCTION

γ-Glutamyl transpeptidase (GGT; EC 2.3.2.2) is a membrane-bound enzyme which catalyzes the hydrolysis of γ-glutamyl compounds and the transfer of γ-glutamyl residues from either glutathione or donor peptides to aa and acceptor peptides (Meister and Anderson, 1983; Orlowski and Meister, 1970). GGT was reported to be involved in detoxification processes (Meister and Tate, 1976) and genesis of ammonia (Tate and Meister, 1975). Continuous administration of ethanol (Ishii et al., 1978) or phenobarbital (Roomi and Goldberg, 1981; Kitagawa et al., 1980) and portacaval shunt (Colombo and Gigon, 1979) lead to increases in GGT activities in the liver. It was also reported that the level of GGT activity in fetal and neonatal liver was much higher than that in adult liver (Albert et al., 1970). In spite of all of these works, the physiological role of GGT in liver particularly during fetal liver development is not known. Systematic studies of gene expression during development should help understand it.

Recently, Sakamuro et al. (1988) cloned human *GGT* cDNA in the fetal liver and deduced the primary structure of the enzyme by determining the nt sequence of the cDNA. It is now possible to study the gene expression of *GGT* both by assaying mRNA and immunohistochemically using mAbs against the purified enzyme. The aim of the present study was to measure *GGT* mRNA and to determine the

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Abbreviations: aa, amino acid(s); ABC, avidin-biotin-peroxidase complex; bp, base pair(s); BSA, bovine serum albumin; GGT, γ-glutamyl transpeptidase; *GGT*, gene (DNA) encoding GGT; kb, kilobase(s) or 1000 bp; mAb, monoclonal antibody; mRNA, messenger RNA; PBS, 0.01 M phosphate buffer containing 0.85% NaCl pH 7.4; r, ribosomal.

amount and location of GGT protein in human liver during fetal development to understand the possible role of GGT in liver development.

EXPERIMENTAL AND DISCUSSION

(a) Detection of GGT mRNA in human fetal liver and its change during embryonic development

Human embryonic tissues were obtained from elective abortion cases at the time of surgical resection. The gestational stage was estimated by the record of conception and the data of ultrasound examinations. Poly(A)$^+$ RNA was isolated from human fetal liver samples taken at various gestation stages and then hybridized with human *GGT* cDNA labeled with ^{32}P to detect *GGT* mRNA. Fig. 1A shows that 2.7-kb mRNA was detected in all samples. The size of the mRNA is the same as the human GGT mRNA reported previously (Goodspeed et al., 1989), and indicates that a 1000-nt nontranslatable region is attached to the coding region for a complete *GGT* of 1707 bp (569 aa) (Sakamuro et al., 1988). In addition to the major mRNA species, a minor band of 1.9 kb is seen in some of the samples, 24- and 40-week-old livers and carcinoma cells, where production of GGT is relatively high. Although mobility of the mRNA species is the same as that of 18S rRNA, it is unlikely that the band is due to cross-hybridization of the probe to the abundant rRNA because the same level of rRNA is present in all samples including the 12-week-old fetal liver.

Change in the level of mRNA during development of the fetal liver was quantified by measuring the density of the autoradiograms shown in Fig. 1 by densitometric scanning. Fig. 2 shows that the relative amount of the mRNA increases gradually as the liver developed to reach a maximum level at week 40 of gestation, just before birth. Due to limitation in obtaining samples, we could not study further changes of *GGT* mRNA in human liver after birth. As a control experiment, amount of mRNA for human γ-actin gene was determined for these poly(A)$^+$ RNA (Fig. 1B). All samples showed a positive band of equal intensity indicating that the actin gene was expressed evenly in the liver during embryonic development. These results strongly suggest that differential expression of *GGT* occurs during development of the human fetal liver. It should be noted that the levels of *GGT* expression in fetal livers are much higher than that in adult liver (Fig. 1A, lane N and Fig. 2, column N) even at week 12 of gestation.

(b) Expression in hepatocarcinoma as compared with the fetal liver

Figs. 1 and 2 also show size and amount of *GGT* mRNA in a hepatocarcinoma tissue and HepG2, a hepatoblastoma cell line, as compared with that in an adult liver harboring a slight fatty metamorphosis. The sizes of the mRNAs in these samples are identical to each other and to mRNA identified in fetal livers. The same sized mRNA was also detected in human fetal kidney at week 24 of gestation (Fig. 3). This is inconsistent with the report by Goodspeed et al. (1989) who showed that the size of *GGT* mRNA from hepatocarcinoma was slightly longer than that from human kidney. The amount of mRNA in the hepatocarcinoma tissue examined here is much higher than that in normal

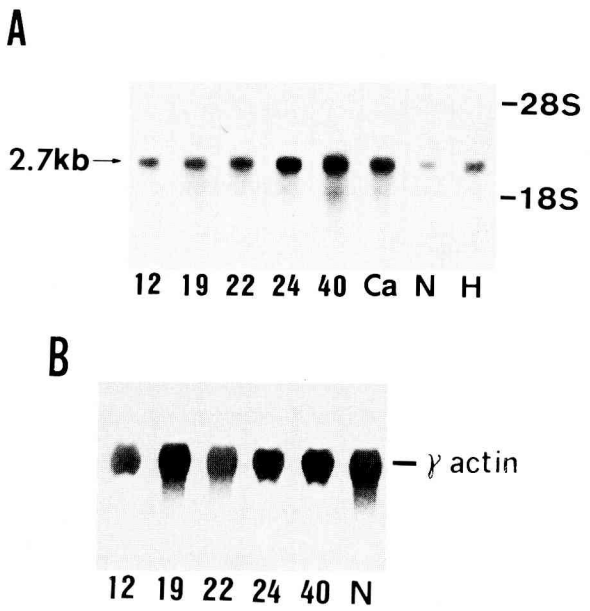

Fig. 1. Detection of mRNA for GGT in fetal liver and other liver cells. Total RNA was extracted by homogenizing tissue samples in guanidine thiocyanate and was further purified by sedimentation through a layer of 5.7 M CsCl as described (Chirgwin et al., 1979). The poly(A)$^+$ RNA was obtained by passing the total RNA through an oligo(dT) cellulose column (Aviv et al., 1972). After ethanol precipitation the RNA was dissolved in 10 mM Tris · HCl/1 mM EDTA pH 7.4. Poly(A)$^+$ RNA (10 μg/ml) was electrophoresed in 1% agarose gels followed by transfer to nitrocellulose filters according to the method of Lehrach et al. (1977). The filter was hybridized to a ^{32}P-labeled single-stranded DNA probe. The following DNA fragments were used as probes: human *GGT*, a 1866-bp *Eco*RI fragment of the human *GGT* cDNA (Sakamuro et al., 1988); γ-actin, a 184-bp *Hin*cII-*Xba*I fragment prepared from pS12 (Emi et al., 1988). The fragments were purified and labeled by the random-priming method (Maniatis et al., 1982) using [α-^{32}P]dCTP(6000 Ci/mmol, Amersham). Probes having specific activities higher than 10^8 cpm/μg DNA were used. (Panel **A**) Numbers underneath the lanes indicate week of gestation of the fetal liver samples. Other lanes are: Ca, a human hepatocarcinoma tissue obtained during surgical operations (provided by the Second Department of Surgical Medicine, Hyogo College of Medicine); N, a human adult liver harboring slightly fatty degeneration; H, HepG2 cells, derived from a human hepatoblastoma (Aden et al., 1979). They were grown in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal calf serum and stored at −70°C until use. (Panel **B**) As controls, the same RNA samples as above were hybridized with γ-actin cDNA. The size of the bands, 2.7 kb, indicated by an arrow was estimated from the position of rRNAs.

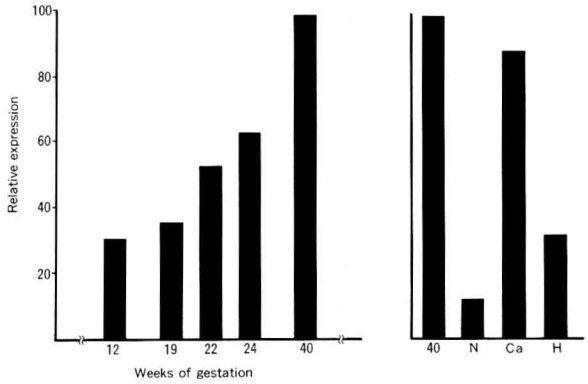

Fig. 2. Amount of the *GGT* mRNA in fetal liver and other liver cells. Autoradiograms of the Northern-blot analysis as shown in Fig. 1 were scanned by densitometer to measure the density of the band (2.7 kb) hybridized to cDNA of *GGT*. Relative amount of mRNA represented by black columns was estimated from the density normalized by the density of the highest level of expression, the fetal liver sample of week 40 of gestation (100%).

adult liver and, interestingly enough, about the same as the maximal level in fetal liver. These results suggest that GGT plays a similar role in developing liver as well as in proliferating carcinoma cells.

(c) Localization of human GGT in fetal livers examined immunohistochemically using mAb

In human liver, GGT is known to be located mainly in regions along the wall of bile canaliculi (Fig. 4A; Albert et al., 1961; Shiozawa et al., 1989). The data presented above demonstrated that expression of the *GGT* gene occurred in fetal liver as early as week 12 of gestation and gradually increased severalfold during embryonic development. To understand the role of GGT in liver, it is essential to elucidate the location of the enzyme protein in fetal liver and its changes during development in parallel with the increase in gene expression. We performed immunohistochemical analyses using a highly specific mAb against purified GGT from human kidney (Shiozawa et al., 1989). This method of precipitation of GGT by mAb is advantageous over the frequently used technique of enzyme histochemistry (Albert et al., 1964; Fleming et al., 1977; Kugler et al., 1985) in determining the precise location of GGT because the final reaction product in enzyme histochemistry is soluble and therefore tends to diffuse away from the site of original deposition.

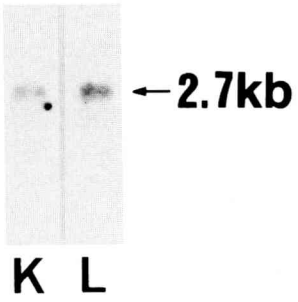

Fig. 3. Detection of GGT mRNA in human fetal kidney and liver. Poly(A)$^+$ RNA (2 μg) was electrophoresed in 1% agarose gel and used for the Northern-blot analysis. Experimental procedures were as described in Fig. 1. Lanes: K, fetal kidney at week 24 of gestation; L, fetal liver at week 24 of gestation.

Fig. 4B shows that distribution of GGT in fetal liver at an early stage of development, at week 8 of gestation, is strictly limited to the wall of bile canaliculi. The intensity of immunostaining of GGT in the wall of bile canaliculi increased in later stages of development, i.e., at week 15 of gestation (Fig. 4C). In even later stages of development, at week 24 of gestation, GGT was found not only in the wall of bile canaliculi, but also all over the membrane of the liver cell (Fig. 4D). Similar distribution of GGT was demonstrated also in the proximal convoluted tubule of the kidney where *GGT* expression is as high as in the fetal liver at later stages of embryonic development (Shiozawa et al., 1989). It seems to be common in many organs that distribution of GGT changes from the apical cell membrane to all over the cell membrane of the tissues as gene expression increases. Such changes, both in amount and distribution of the enzyme, may be related to the physiological change of cells during development of tissues and organs.

Previous studies with rat liver showed that GGT in the fetal liver was found only in the last one third of gestation (Hanigan and Pitot, 1985). Similar transient production of GGT was also observed during development of other tissues (Menard et al., 1981; Lisy et al., 1981; Richards and Astrup, 1982). In contrast, our present results with human fetal liver clearly demonstrated continuous and increasing expression of *GGT* from the first trimester through the third trimester of gestation. The difference in gene expression between rat and human may be attributable to the difference in the function of GGT in fetal hepatocytes of the two species. Further studies are required to elucidate the biological role of GGT in human liver.

(d) Conclusions

(1) Expression of human *GGT* gene in liver was examined using Northern analysis by cDNA as a probe during fetal liver development. The expression was detectable as early as at week 12 of gestation and then increased gradually to a peak at week 40, just before birth. The maximal level of the expression in the fetal liver was tenfold higher than that in adult liver and comparable to that in a hepatocellular carcinoma.

(2) Judging from the size (2.7 kb), the same species of mRNA was produced in human fetal liver, human fetal kidney and human hepatocellular carcinoma.

(3) Production and localization of GGT in liver were

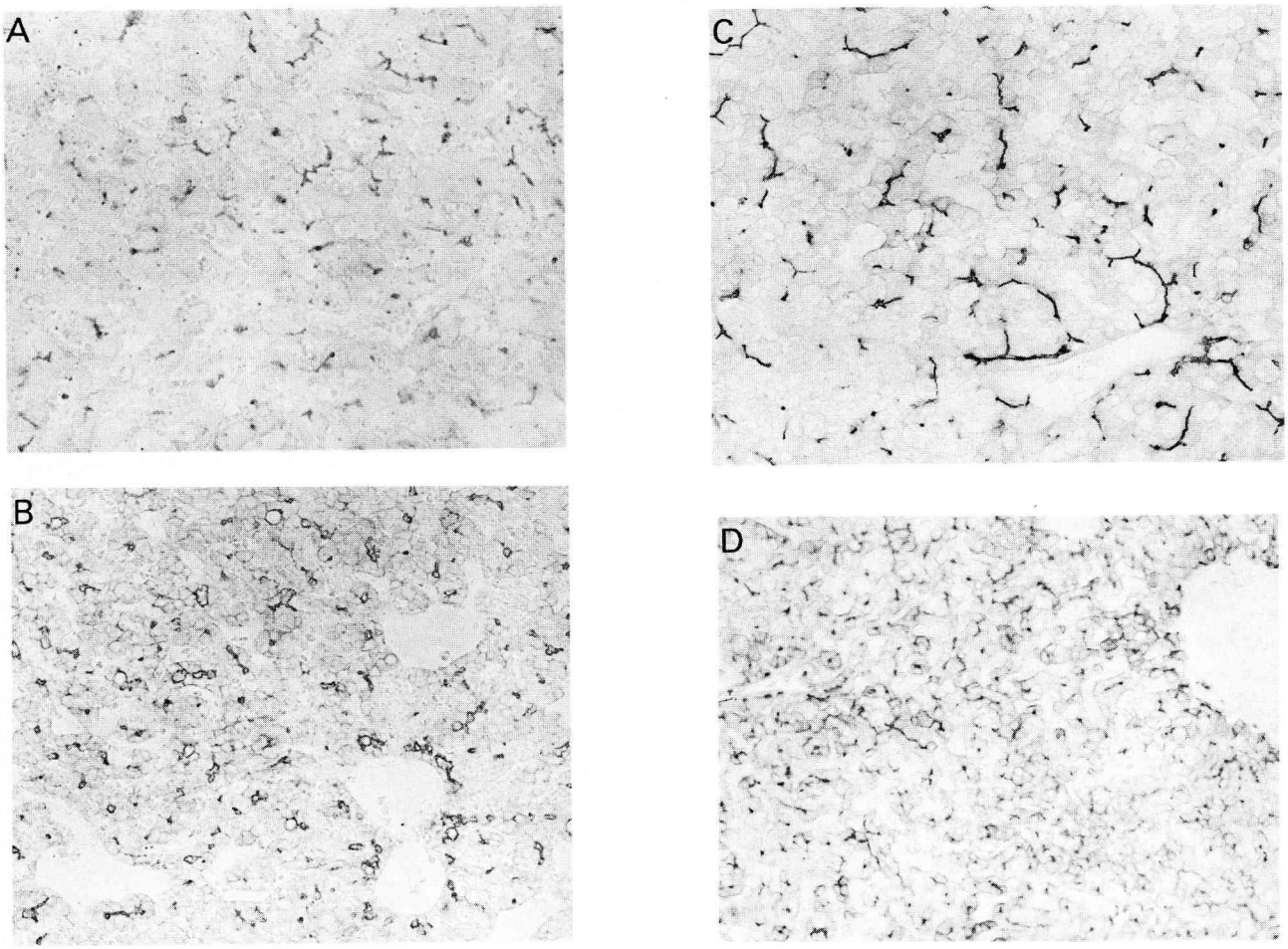

Fig. 4. Immunohistochemical staining of human GGT in liver at various developmental stages. Small blocks of liver were fixed with 4% paraformaldehyde, dehydrated in an ascending series of ethanol, cleaned in xylene, and embedded in paraffin. Tissue sections of 4 μm thick were prepared. They were deparaffinized and washed with PBS. For immunostaining, sections were treated with 1% BSA dissolved in PBS and then with anti-human GGT mAb (Shiozawa et al., 1989) for 2 h at room temperature. After being washed with PBS, the sections were stained by the ABC procedure (Hsu et al., 1981) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). They were then treated in a diaminobenzidine solution containing 25 mg 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) and 0.25 ml of 1% H_2O_2 in 100 ml of 0.05 M Tris · HCl buffer pH 7.4. After the immunostaining, paraffin sections were counterstained with 0.1% methyl green solution, dehydrated, cleaned in xylene and mounted in balsam. For control experiments, the following three procedures were performed. (a) The first antibody was omitted; (b) the first antibody was replaced by an irrelevant mAb against human prostatic acid phosphatase (Cosmo Bio Co., Tokyo, Japan); (c) the ABC procedure was omitted. (**A**) A sample of adult liver with slight metamorphosis used for mRNA measurement (lane N in Fig. 1A and column N in Fig. 2) was used for immunohistochemical staining. A weak reaction was observed in the wall of bile canaliculi. (**B**) Liver at week 8 of gestation. Positive reaction was observed along the wall of the bile canaliculi. (**C**) Liver at week 15 of gestation. Positive reaction was observed distinctively along the wall of bile canaliculi. (**D**) Liver at week 24 of gestation. Positive reaction was observed not only in the wall of the bile canaliculi, but also all over the cell membrane of liver cells.

determined histochemically using mAb against human GGT. In parallel to the increase in mRNA synthesis, increase in both areas and intensity of immunostaining was observed. Furthermore, GGT protein was shown to be localized restrictively in the wall of bile canaliculi when the expression was low, but distributed widely over all along the cell membrane of the fetal hepatocytes when the expression was high.

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The primary structure of human gamma-glutamyl transpeptidase

(Recombinant DNA; cDNA; nucleotide sequence; amino acid sequence; human fetal liver; human kidney; rat enzyme)

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SUMMARY

A cDNA hybridizable to that of rat γ-glutamyl transpeptidase (GGT) was cloned from a cDNA library of human fetal liver. The insert of the cDNA clone contained 1866 bp consisting of an open reading frame (ORF) of 1709 bp (569 amino acids (aa), N-terminal portion truncated) and a 135-bp 3′-untranslated region followed by a polyadenylated tail. In parallel, amino acid sequences of N-terminal portions of heavy and light chains of a purified human GGT were determined. Two stretches of amino acid sequences identical to the N-terminal sequences of heavy and light chains were found in the ORF. We therefore concluded that the clone is a cDNA for human GGT. From the amino acid sequence deduced from cDNA, the heavy and the light chains of the purified enzyme are estimated to be composed of 351 aa (M_r 38336) and of 189 aa (M_r 20000), respectively. The heavy chain is preceded by a signal peptide of at least 29 aa presumed to be cleaved by bromelain treatment. Six putative *N*-glycosylation sites are present in the heavy subunit region and one in the light subunit region. Primary structure and hydrophobicity profile are closely similar to those of rat GGT.

INTRODUCTION

The GGT, a glycoprotein of *N*-glycoside type, catalyzes hydrolysis of glutathione and transfer of the γ-glutamyl residue and is widely distributed in mammalian tissues (Meister et al., 1981). The enzyme is used as an important marker enzyme for chemically induced hepatoma, since it is induced in primary hepatoma and preneoplastic lesions of the liver that are thought to be responsible for this

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Abbreviations: aa, amino acid(s); bp, base pair(s); GGT, γ-glutamyl transpeptidase; HPLC, high-performance liquid chromatography; kb, kilobase(s) or 1000 bp; LDS, lithium dodecyl sulfate; nt, nucleotide(s); ORF, open reading frame; PTH, phenylthiohydantoin.

neoplasm (Fiala et al., 1972; Taniguchi, 1974; Cameron et al., 1978). We have isolated and characterized rat GGTs from various tissues: an azo dye-induced hepatoma (Taniguchi, 1974), fetal liver (Taniguchi et al., 1975), AH-66 ascites hepatoma and yolk-sac tumors (Yokosawa et al., 1981). We reported that the enzymes from different sources were similar in antigenicity and physicochemical properties (Taniguchi et al., 1985b). Major differences in properties of enzymes from different tissues especially between normal and hepatoma tissues occur in their carbohydrate moieties (Taniguchi et al., 1985b). The GGT from AH-66 hepatoma contains a unique carbohydrate structure (Yamashita et al., 1983) which is closely associated with the induction of one of the glycosyltransferases (Nishikawa et al., 1988).

We have shown previously that GGT in sera of patients with primary hepatoma can be used as a useful marker for monitoring and diagnosis using immunochemical techniques (Taniguchi et al., 1985a,b). However, little is known about the primary structure of the human enzyme except for the fact that purified enzyme consists of two peptide chains, heavy and light, similar in size to those of the rat enzyme. Since we have succeeded in preparing a highly purified enzyme from human kidney and cDNA of the rat enzyme (Coloma and Pitot, 1986; Laperche et al., 1986) has become available, we determined the primary structure of the human enzyme. We found that the partial amino acid sequence determined for the purified enzyme completely agreed with the amino acid sequence deduced from the nucleotide sequence of the cDNA which was cloned by cross hybridization with the cDNA of the rat GGT. The primary structure of the human enzyme is compared with that of the rat enzyme.

MATERIALS AND METHODS

(a) cDNA

A cDNA library of human fetal liver prepared in λgt11 was purchased from Clontech Laboratories Inc., Palo Alto, CA (Cat. No. HL1005, Lot 0128). Two cDNA clones, 13I and 10I, gifts from Dr. H.C. Pitot (University of Wisconsin, Madison, WI) contain cDNA inserts corresponding to mRNA of heavy and light subunits of the rat GGT, respectively (Coloma and Pitot, 1986).

(b) Chemicals

Restriction endonucleases, other enzymes for recombinant DNA techniques and M13 sequencing kits were from Takara Shuzo Co., Kyoto, Japan. [α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham, Bucks., U.K.

(c) Recombinant DNA techniques

Screening of the cDNA library was carried out essentially as described by Maniatis et al. (1982) except that stringency of hybridization was slightly reduced; incubation was performed in 40% (v/v) formamide at 42°C for 2 h. Restriction enzyme fragments of the cDNA and their sequential deletion fragments, prepared using a kit from Takara Shuzo Co. (Kyoto, Japan), were subcloned into bacteriophage vectors M13mp18 and M13mp19. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977).

(d) Purification of mature gamma-glutamyl transpeptidase from human kidney and its microsequence analysis

Human kidney GGT was purified as described previously (Taniguchi et al., 1985b) using luburol and bromelain treatments followed by immunoaffinity chromatography. The purified enzyme was dissolved in 1% LDS containing 0.5% 2-mercaptoethanol, heated at 100°C for 3 min and then applied to a gel-permeation HPLC with TSKgel G3000SW. Elution with an 100 mM NH_4 · succinate buffer (pH 6.8), containing 0.03% LDS resulted in separation of the enzyme in two fractions, the heavy and light subunits. Each peak was lyophilized and subjected to microsequence analysis on a pmol scale using a gas-phase sequencer, Model 470A (Applied Biosystems, San Francisco, CA). The resulting PTH-aa were analyzed by the reverse-phase HPLC system, Model 120A (Applied Biosystems, San Francisco, CA).

RESULTS AND DISCUSSION

(a) Partial amino acid sequence of heavy and light subunits of human gamma-glutamyl transpeptidase

Sequences of 29 and 28 aa from N termini of, respectively, the heavy and light subunits of the purified enzyme were determined by automated microsequence analysis. From the data in Fig. 1, the sequences for the heavy and light subunits were determined as: Ser-Lys-Glu-Pro-Asp-Asn-His-Val-Tyr-Thr-Arg-Ala-Ala-Val-Ala-Ala-Asp-Ala-Lys-Gln-X-Ser-Lys-Ile-Gly-Arg-Asp-Ala-Leu and Thr-Ala-His-Leu-Ser-Val-Val-Ala-Glu-Asp-Gly-Ser-Ala-Val-Ser-Ala-Thr-Ser-Thr-Ile-Asn-Leu-Tyr-Phe-Gly-Ser-Lys-Val. The heavy chain of the enzyme is known to contain a stretch of hydrophobic amino acids at the N-terminal portion which is susceptible for cleavage by the bromelain treatment (Matsuda et al., 1983). Since the enzyme used in this study was treated by bromelain, the purified enzyme was presumed to have lost this hydrophobic stretch from the heavy subunit. The comparison of the N-terminal sequence with the sequence of the peptide coded by cDNA (see RESULTS AND DISCUSSION, section **c**) revealed that this was indeed so.

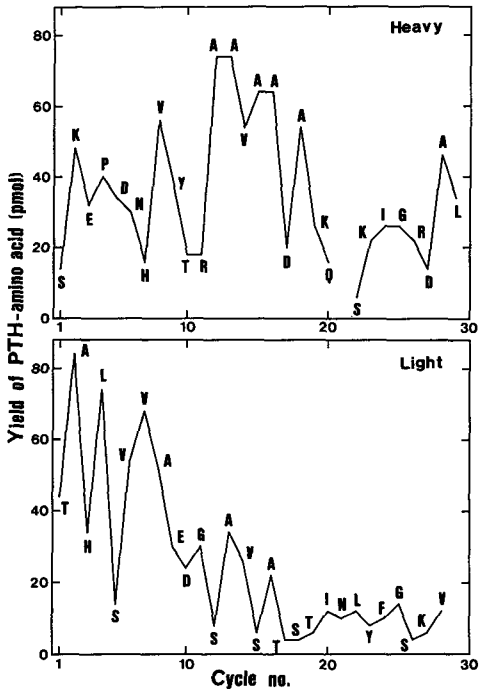

Fig. 1. Partial amino acid sequence of heavy (panel **Heavy**) and light (panel **Light**) subunits of human GGT. Each subunit of the purified enzyme (see MATERIALS AND METHODS, section **d**) was applied to a gas-phase sequencer on a pmol scale and phenylthiohydantoin (PTH) derivatives of aa were analyzed by HPLC. The maximal yield of PTH amino acids at each cycle of Edman degradation is shown. One-letter amino acid notation is used.

(b) Isolation of putative human gamma-glutamyl transpeptidase cDNA clones

Two cDNA clones, 13I and 10I, of the rat GGT (see MATERIALS AND METHODS, section **a**) were used to screen 6.0×10^5 recombinant clones from a human fetal liver cDNA library constructed in λgt11 to isolate a human counterpart of the rat cDNA for GGT. Three clones, c1A, c2A and c3B, hybridized equally well with both 13I and 10I probes and were found to contain inserts of the same size of 1.9 kb.

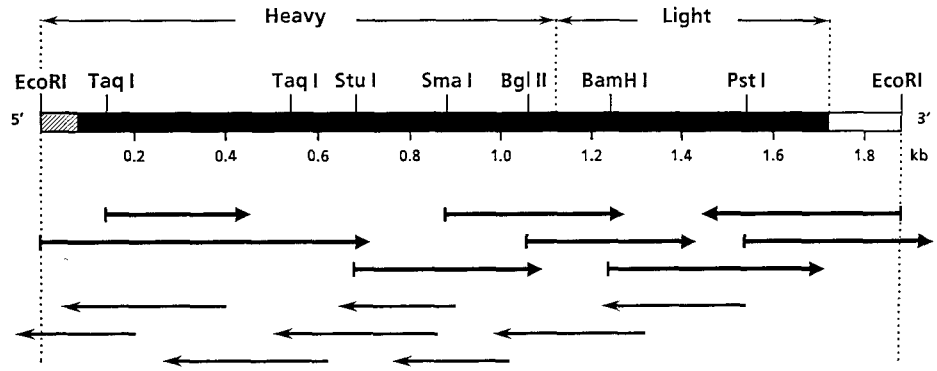

Fig. 2. Restriction map and sequencing strategy for cDNA of human GGT. Relevant restriction sites are indicated. The solid bar region represents 1620 nt that code the 540 aa of the purified enzyme treated by bromelain. The hatched region denotes the putative signal peptide region of 29 aa. The open region is 157 nt 3'-untranslated sequence. Below the map, arrows indicate direction and extent of sequencing either of sucloned restriction fragments (thick arrows) or of a series of deletion fragments prepared by exonuclease III (thin arrows), respectively. 'Heavy' and 'Light' are heavy and light subunits of human GGT, respectively.

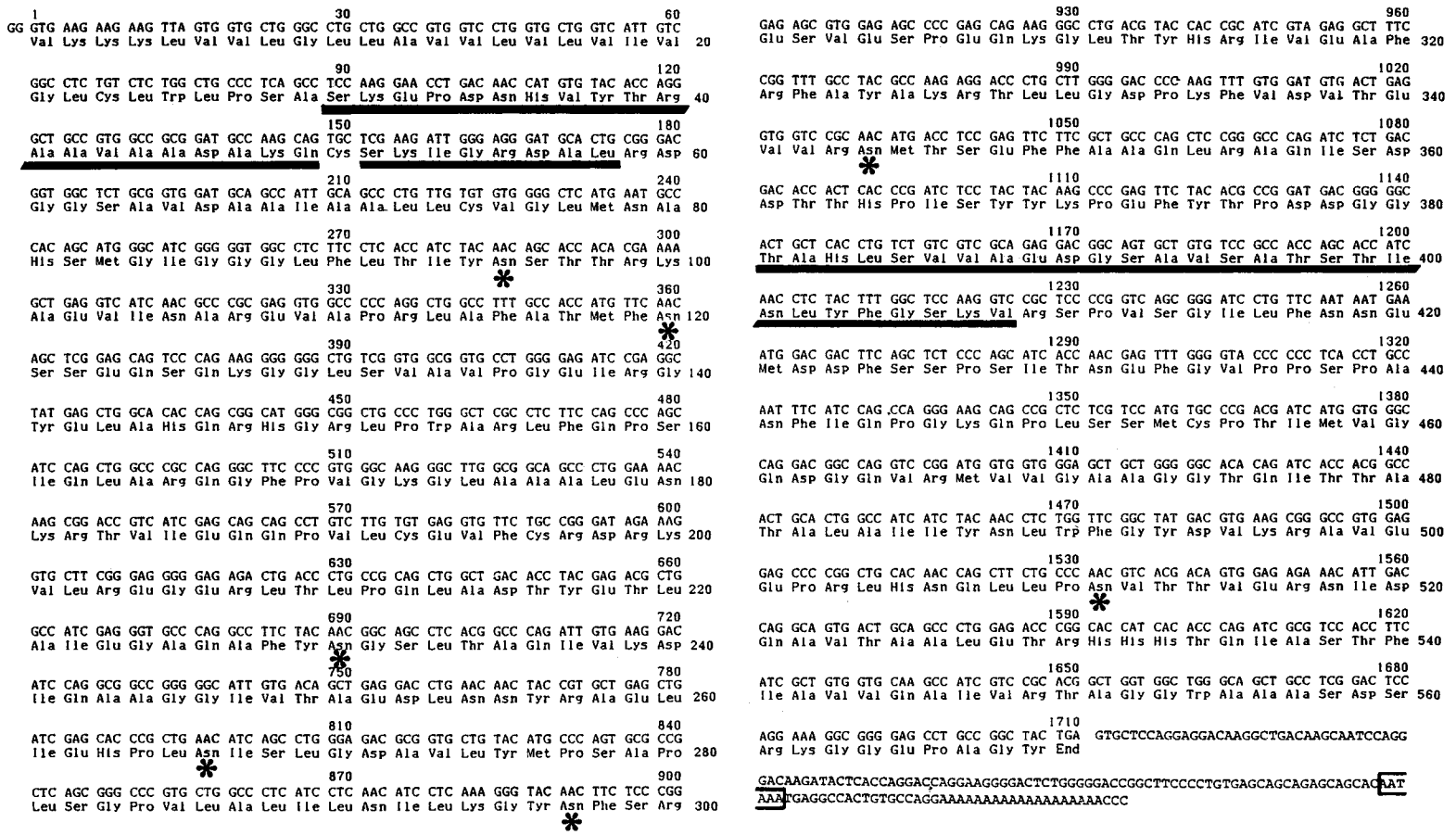

Fig. 3. Nucleotide sequence of the cDNA and amino acid sequence of an ORF deduced from it. The nt = 1 corresponds to the first nt of the first codon identified in the ORF. Amino acid sequences identical with chemically determined amino acid sequences of N-terminal portions of heavy (aa 30–58 except for Cys-50) and light (aa 381–408) subunits of the purified enzyme are heavily underlined. Putative *N*-glycosylation sites are indicated by asterisks under the aa. Presumed polyadenylation signal AATAAA is boxed.

Nucleotide sequences of about 400 bp from both 5′ and 3′ ends of the inserts revealed that the three clones were identical.

(c) Nucleotide sequence of cDNA and deduced amino acid sequence

The complete nucleotide sequence of the clone c2A was determined according to the strategy shown in Fig. 2. It contains a 1866-bp insert excluding *Eco*RI linkers, with a long ORF of 1709 bp which is truncated at the 5′ end and terminated by a TGA stop codon (Fig. 3). The 3′-untranslated region is 157 bp long including a consensus signal (Proudfoot and Brownlee, 1976) for polyadenylation followed by poly(A). Two stretches of amino acid sequence within the ORF (aa 30–58 and aa 381–408; underlined in Fig. 3) are identical to chemically determined sequences of N-terminal portion of the heavy and light subunits of the purified enzyme, respectively (see RESULTS AND DISCUSSION, section **a**). These results indicate conclusively that the clone c2A is the cDNA of the mRNA for human GGT.

The sequence data of the cDNA also reveals that two subunits of the mature enzyme are the products of processing of the single precursor peptide. This was suggested previously in rat GGT by biochemical evidences (Nash and Tate, 1982; Yokosawa et al., 1983; Finidori et al., 1984), though there is no evidence that such occurs in human GGT. If there is no loss of amino acids during processing, the heavy subunit is estimated to be 351 aa (M_r 38 336) and the light subunit 189 aa (M_r 20 000). The heavy subunit is preceded by a peptide of at least 29 aa, the true length of which is not known at present because the 5′ end of the insert is terminated with two odd nucleotides. The remarkably high hydrophobic property of this peptide suggests that it is the leader peptide which anchors the enzyme to the cytoplasmic membrane and is cleaved by the bromelain treatment. The C-terminal amino acid of the peptide is Ala which is often found as the C-terminal residue of the known signal peptides (Heijne, 1983).

(d) Homology between human and rat gamma-glutamyl transpeptidases

The comparison of the primary sequence of the human enzyme with those of the rat enzyme reported previously (Laperche et al., 1986; Coloma and Pitot,

```
                                  70                                  80
Residues   Val Asp Ala Ala Ile Ala Ser Leu Leu Cys Met Gly Leu Ile Asn Ala His Ser
a;         GTG GAC GCG GCC ATC GCA AGC CTG CTG TGT ATG GGG CTC ATT AAT GCC CAC AGT
b;         GTG GAC G*GG CCA TCG CAA GCC TGC TGT GTA TGG GGC TCA TTA ATG CCC ACA GTA
c;         GTG GAC GCG GCC ATC GCA *GCC TGC TGT GTA TGG GGACTC ATT AAT GCC CAC *GTA
                                                    *

                                      90                                  100
Residues   Met Gly Ile Gly Gly Gly Leu Phe Phe Thr Ile Tyr Asn Ser Thr Thr Arg Lys
a;         ATG GGC ATC GGG GGC GGC CTC TTC TTC ACC ATC TAC AAC AGC ACC ACA CGA AAA
b;          TGG GCA TCG GGG GCG GCC TCT TCT TCA CCA TCT ACA ACA GCA CCA CAC GAA AAG
c;          TGG GCA TCG GGG GCG GCC TCT TCT TCA CCA TCT ACA ACA GCA CCA CAC GAA AAG

                                     110
Residues   Ala Glu Val Ile Asn Ala Arg Glu Met Ala Pro Arg Leu Ala Asn Thr Ser Met
a;         GCT GAA GTT ATC AAT GCC CGT GAA ATG GCT CCC AGG TTG GCC AAT ACC AGC ATG
b;          CTG AAG TTA TCA ATG CCC GTG AAA TGG CTC CCA GGT TGG CCA ATA CCA GCA TGT
c;          CTG AAG TTA TCA ATG CCC GTG AAA TGG CTC CCA AGT TGG CCA ATA CCA GCA TGT
                                                    *
    120                                 130
Residues   Phe Asn Asn Ser Lys Asp Ser Glu Glu Gly Gly Leu Ser Val Ala Val Pro Gly Glu
a;         TTC AAT AAT TCT AAG GAC TCT GAA GAA GGA GGC CTT TCA GTG GCA GTT CCT*GGT GAA
b;          TCA ATA ATT CTA AGG ACT CTG AAG AAG GAG GCC TTT CAG TGG CAG TTC CTTGGT GAA
c;          TCA ATA ATT CTA AGG ACT CTG AAG AAG GAG GCC TTT CAG TGG CAG TTC CTTGGT GAA
                                                                          *
```

Fig. 4. Reexamination of nucleotide sequence of the rat GGT cDNA. The nucleotide sequence of the portion of rat GGT cDNA, 13I, corresponding to aa 48–161 was reexamined. The data shown in line (**a**) are compared with two previously reported sequences, (**b**) by Coloma and Pitot (1986) and (**c**) by Laperche et al. (1986). Sites of discrepancy from the present results are marked with asterisks. In (**b**) one deletion and one insertion error are observed. In (**c**) two deletions errors, two insertions errors, and one substitution error are observed. The corrected amino acid sequence, which is in frame with the reported rat GGT, was deduced from the present nucleotide sequence. In both (**b**) and (**c**), amino acid sequences deduced from the nucleotide sequence marked with underlines are different from the corresponding amino acid sequences obtained in this experiment because of frame shift due to the deletion or insertion errors.

1986) reveals that they are remarkably similar and suggests a close evolutionary relation. After close examination, however, we found that a stretch of amino acids of the human enzyme (aa 67–135) had essentially no similarity to the corresponding region of the rat enzyme. Because of its extraordinary dissimilarity, we have reexamined this portion of rat cDNA, clone 13I, and found that the reported sequence contained a few single base deletions and insertions in this region (Fig. 4). The revised sequence is highly similar to that of the human enzyme. The sequence of this region was also reexamined in Pitot's laboratory independently and the results agreed with ours (H.C. Pitot, personal communication). Using the corrected data, amino acid sequence similarity between human and rat GGTs is estimated

```
                10         20         30         40         50         60
Human ; VKKKLVVLGLLAVVLVLVIVGLCLWLPSASKEPDNHVYTRAAVAADAKQCSKIGRDALRD
          :    :::: ::::: :: ::: :::  :  :: ::: ::::: ::: :: :::: :
Rat   ; MKNRFLVLGLVAVVLVFVIIGLCIWLPTTSGKPD HVYSRAAVATDAKRCSEIGRDMLQE

                70         80         90        100        110        120
Human ; GGSAVDAAIAALLCVGLMNAHSMGIGGGLFLTIYNSTTRKAEVINAREVAPRLAFATMFN
          ::: :::::: ::: :: ::::::::::::: :::::::::::::::::: :::::   :::
Rat   ; GGSVVDAAIASLLCMGLINAHSMGIGGGLFFTIYNSTTRKAEVINAREMAPRLANTSMFN

               130        140        150        160        170        180
Human ; SSEQSQKGGLSVAVPGEIRGYELAHQRHGRLPWARLFQPSIQLARQGFPVGKGLAAALEN
          :  : ::::::::::::::::::::::::::::::::::::::::: ::::::::: ::
Rat   ; NSKDSEEGGLSVAVPGEIRGYELAHQRHGRLPWARLFQPSIQLARHGFPVGKGLARALDK

               190        200        210        220        230        240
Human ; KRTVIEQQPVLCEVFCRDRKVLREGERLTLPQLADTYETLAIEGAQAFYNGSLTAQIVKD
          ::  ::  : :::::::  ::: ::: : : ::::   :: ::: ::::::::::::::
Rat   ; KRDIIEKTPALCEVFCRQGKVLQEGETVTMPKLADTLQILAQEGARAFYNGSLTAQIVKD

               250        260        270        280        290        300
Human ; IQAAGGIVTAEDLNNYRAELIEHPLNISLGDAVLYMPSAPLSGPVLALILNILKGYNFSR
          :: :::: : ::::::::: ::::  : :::  :: :::::::: ::::::::::::
Rat   ; IQEAGGIMTVEDLNNYRAEVIEHPMSIGLGDSTLYVPSAPLSGPVLILILNILKGYNFSP

               310        320        330        340        350        360
Human ; ESVESPEQKGLTYHRIVEAFRFAYAKRTLLGDPKFVDVTEVVRNMTSEFFAAQLRAQISD
          ::  :::: :::::::::::::::::: ::::::::  : ::: ::: : :::: : :
Rat   ; KSVATPEQKALTYHRIVEAFRFAYAKRTMLGDPKFVDVSQVIRNMSSEFYATQLRARITD

               370        380        390        400        410        420
Human ; DTTHPISYYKPEFYTPDDGGTAHLSVVAEDGSAVSATSTINLYFGSKVRSPVSGILFNNE
          :::: ::  ::: :::::::::::: :::::: :::::::::::: : ::::::: :
Rat   ; ETTHPTAYYEAEFYLPDDGGTAHLSVVSEDGSAVAATSTINLYFGSKVLSRVSGILFNDE

               430        440        450        460        470        480
Human ; MDDFSSPSITNEFGVPPSPANFIQPGKQPLSSMCPTIMVGQDGQVRMVVGAAGGTQITTA
          ::::::: :: ::: :::::::  ::::::::: : :  :: ::::::: :::::::
Rat   ; MDDFSSPNFTNQFGVAPSPANFIKLGKQPLSSMCPSIIVDKDGKVRMVVGASGGTQITTS

               490        500        510        520        530        540
Human ; TALAIIYNLWFGYDVKRAVEEPRLHNQLLPNVTTVERNIDQAVTAALETRHHHTQIASTF
          ::::: ::::::::::::::::::::: :::: :::: ::: : ::::::     :
Rat   ; VALAIINSLWFGYDVKRAVEEPRLHNQLLPNTTTVEKNIDQVVTAGLKTRHHHTEVTPDF

               550        560
Human ; IAVVQAIVRTAGGWAAASDSRKGGEPAGY
          :::::: ::: ::::::::::::::::
Rat   ; IAVVQAVVRTSGGWAAASDSRKGGEPAGY
```

Fig. 5. Similarity in amino acid sequence between human and rat GGTs. The amino acid sequence (denoted by single-letter code) deduced from cDNA of human GGT was aligned with that of rat GGT to obtain maximal similarity (colons specify the identical aa). For the rat sequence, the data of Laperche et al. (1986) were used except for the reexamined region shown in Fig. 4. The similarity is 78.9%. Consensus N-glycosylation sites are marked with asterisks (above aa for human and below aa for rat sequences).

to be 79% with only one aa deletion in the rat enzyme (Fig. 5). Variations of amino acids are scattered more or less randomly with significantly higher variations in the N-terminal region. Although N-terminal Met is identified in the rat enzyme, the counterpart of the human enzyme may be further extended as discussed previously (RESULTS AND DISCUSSION, section **c**). The hydrophobicity plot of the human enzyme is also very similar to that of the corrected sequence of the rat enzyme (Fig. 6).

(e) The possible sites of *N*-glycosylation

If one takes Asn-X-Thr and Asn-X-Ser as consensus sequences for *N*-glycosylation sites, six and one possible sites are found in heavy and light subunits of the human enzyme, respectively (Fig. 3). Among these sites at least six, Asn-95, -120, -230, -266, -297, and -511, can be glycosylated readily because they are located in hydrophilic regions (Fig. 6). The heavy and light subunits of the purified enzyme from human kidney prepared by the bromelain treatment have M_rs of 62000 and 22000, respectively (Taniguchi et al., 1985b). On the other hand their M_rs calculated from the sequence data were 38336 and 20000, respectively (RESULTS AND DISCUSSION, section **c**). This discrepancy can be explained if the mature enzyme is fully glycosylated: six residues in the heavy and one in the light subunit,

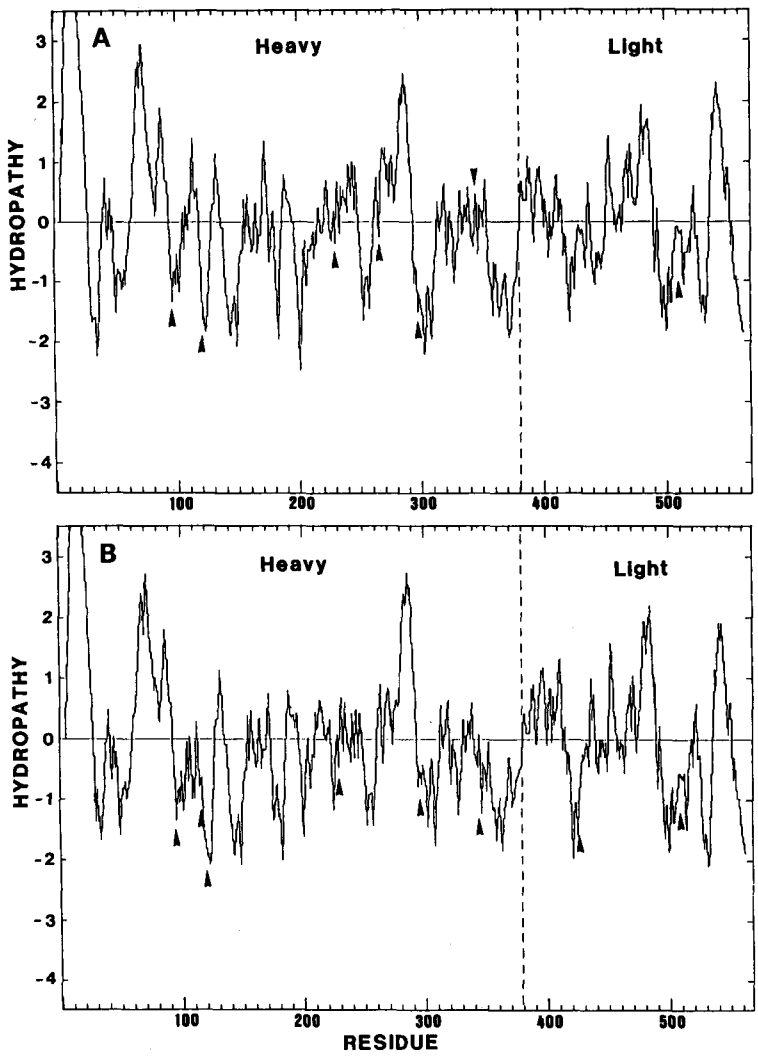

Fig. 6. Hydropathy profiles of human (**A**) and rat (**B**) GGTs. Hydropathy scores were calculated by the method of Kyte and Doolittle (1982), using a window size of 11 aa. Hydrophobicity and hydrophilicity increase with distance above and below zero, respectively. The corrected data in Fig. 5 were used for the rat profile. Putative *N*-glycosylation sites are shown by arrowheads.

assuming that each *N*-linked carbohydrate moiety has an M_r of 2000–3000.

The number of possible glycosylation sites was five in the rat enzyme deduced from the reported sequence of rat cDNA, and it seems to be too few considering the fact that the level of glycosylation of the enzyme from AH-66 rat ascites hepatoma is four times as much as that of the enzyme from normal rat liver (Yamashita et al., 1983). The revised sequence revealed three additional glycosylation sites in the rat enzyme (Figs. 5 and 6B).

(f) The active site of gamma-glutamyl transpeptidase

The active site of GGT is known to be located in the light subunit of the mature enzyme (Tate and Meister, 1977). The enzyme is known to bind with γ-glutamyl residue during hydrolysis of glutathione. In addition the enzyme has a phosphate-independent glutaminase activity (Tate and Meister, 1975), suggesting the presence of a glutamine-binding site as well. Since the primary structures of human and rat enzymes are now available, we tried to locate possible active sites in the light subunit using the information on amino acid sequence of active sites of the closely related enzymes. Several glutamine amido-*N*-transferases, anthranilate synthase (Kawamura et al., 1978; Tso et al., 1980; Paluh et al., 1985), carbamyl phosphate synthetase (Rubino et al., 1986) and CTP synthetase (Weng et al., 1987), are similar in function, especially both in binding covalently with the glutamine residue and exerting glutaminase activity. A highly conserved sequence containing a cysteine flanked by hydrophobic amino acids was found at the glutamine-binding sites of these enzymes (Table I). Although no general similarity in amino acid sequence was detected between GGTs and these glutamine amido-*N*-transferases, a stretch of amino acids in the light subunit of the two GGTs containing the single cysteine showed a weak similarity with the glutamine-binding sequence (Table I).

These results suggest that the active site of both human and rat GGT is located at or near the single cysteine residue in the light subunit and binds with both glutamine and γ-glutamyl residues.

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TABLE I

Amino acid sequence of putative glutamate or glutamine-binding site of GGT and other related enzymes

Enzyme[a]	Residues (aa)[b]	Sequence (aa)[c]	References
Human GGT	446–461	GKQPLSSMCPTIMVGQ	This work
Rat GGT	445–460	GKQPLSSMCPSIIVDK	Laperche et al. (1986)
P. putida ASII	71– 86	GKLPILGV*CLGHQSIG	Kawamura et al. (1978)
S. marcescens ASII	76– 91	GRLPIIGI*CLGHQAIV	Tso et al. (1980); Paluh et al. (1985)
E. coli CPS	261–276	TDIPVFGI*CLGHQLLA	Rubino et al. (1986)
E. coli CTP synthetase	371–385	NNIPYLGI*CLGMQ VA	Weng et al. (1987)

[a] ASII, anthranilate synthase component II; CPS, carbamyl phosphate synthetase; CTP, cytidine 5′-triphosphate.

[b] The numbering of aa is according to each reference.

[c] The underlined residues indicate identical aa with respect to the human GGT. The asterisks denote the active sites determined by alkylation with iodoacetamide (Kawamura et al., 1978; Tso et al., 1980) and 6-diazo-5-oxo-L-norleucine (Weng et al., 1987) or by site-directed mutagenesis (Paluh et al., 1985; Rubino et al., 1986).

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