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The poliovirus receptor protein is produced both as membrane-bound and secreted forms

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Both genomic and complementary DNA clones encoding poliovirus receptors were isolated from genomic and complementary DNA libraries prepared from HeLa S3 cells, respectively. Nucleotide sequence analysis of these cloned DNAs revealed that the poliovirus receptor gene is ~20 kb long and contains seven introns in the coding region, and that at least four mRNA isoforms referring to the coding sequence are generated by alternative splicing and appear to encode four different molecules, that is, PVRα, PVRβ, PVRγ and PVRδ. The predicted amino acid sequences indicate that PVRα and PVRδ, corresponding to the previously described cDNA clones H20A and H20B, respectively, are integral membrane proteins while the other two molecules described here for the first time lack a putative transmembrane domain. Mouse cell transformants carrying PVRα were permissive for poliovirus infection, but those carrying PVRβ were hardly permissive. In contrast to PVRα, PVRβ was not detected on the surface of the mouse cell transformants but was detected in the culture fluid by an immunological method using a monoclonal antibody against poliovirus receptor. Three types of splicing products for PVRα, PVRβ and PVRγ were detected by polymerase chain reactions using appropriate primers in poly(A)$^+$ RNAs of the brain, leukocyte, liver, lung and placenta of humans; the choice of primers used did not permit detection of PVRδ. *In situ* hybridization using a cDNA fragment as a probe demonstrated that the PVR gene is located at the band q13.1 → q13.2 of human chromosome 19.

Key words: alternative splicing/cellular receptor/poliovirus/secreted form

Introduction

Poliovirus, the causative agent of poliomyelitis, is a human enterovirus that belongs to the Picornaviridae, and is classified into three stable serotypes. Most poliovirus strains infect only primates. Poliovirus infection is initiated by ingestion of virus and its primary multiplication in the oropharyngeal and intestinal mucosa. The tonsils and Peyer's patches of the ileum are invaded early in the course of infection, and extensive viral multiplication occurs in these loci. From the primary site of propagation, virus drains into deep cervical and mesenteric lymph nodes, and then into the blood. If effective viremia is established, virus spreads to the central nervous system. Paralytic poliomyelitis occurs as a result of destruction of motor neurons in the brain and spinal cord by the lytic virus replication (Bodian, 1955; Sabin, 1956). Thus, sites of poliovirus replication are limited to certain tissues, although many tissues are exposed to the virus during the viremic phase.

An important determinant in the development of disease relies on the characteristic tissue tropism of different picornaviruses, which appears to be governed by the accessibility of specific cellular receptors. Indeed, experimental evidence has strongly suggested that the species specificity and tissue tropism of poliovirus are primarily determined by a unique cell surface receptor (Holland *et al.*, 1959; Holland, 1961; Crowell and Landau, 1983). Furthermore, all three serotypes of poliovirus compete for the same cellular receptor which is not recognized by other picornaviruses (Crowell *et al.*, 1987). Thus, studies on the cellular receptor for poliovirus are essential to understand the molecular basis of the disease syndrome.

Several monoclonal antibodies which block cellular receptors for poliovirus infection have been isolated (Minor *et al.*, 1984; Nobis *et al.*, 1985; Shepley *et al.*, 1988). Monoclonal antibody D171 protected HeLa cells against the cytopathic effect (CPE) of all three poliovirus serotypes. This antibody bound specifically to all cell lines of human or primate origin tested (Nobis *et al.*, 1985). Monoclonal antibody AF3 was used as a probe in immunoblots of cell membrane preparations and detected a material with a mol. wt of 100 kd in only those from cell lines and tissues permissive for poliovirus infection (Shepley *et al.*, 1988). This antibody stained specific regions of the brainstem that previously had been shown to be damaged in poliomyelitis.

Although mouse L cells are not permissive for poliovirus infection, the cells transfected with poliovirus RNA produce infectious poliovirus particles. Several years ago, mouse L cell transformants permissive for poliovirus infection were generated by the transformation of mouse L cells with human DNA (Mendelsohn *et al.*, 1986). Recently, cDNA clones (H20A and H20B) encoding functional poliovirus receptors (PVRs) were isolated (Mendelsohn *et al.*, 1989). The amino acid sequences deduced from the nucleotide sequences of the cDNAs revealed that the human PVRs are integral membrane proteins with three domain structures characteristic of members of the immunoglobulin (Ig) superfamily.

Here we describe the structure of the human PVR gene and the existence of secreted forms of PVR molecules that are derived from the same gene by alternative splicing.

Furthermore, both types of mRNAs for membrane-bound and secreted forms are detected by polymerase chain reaction (PCR) experiments in the brain, leukocytes, liver, lung and placenta of humans. We also demonstrate that the PVR gene is located at the band q13.1 → q13.2 of human chromosome 19.

Results

Structure of the PVR gene and multiple splicing of its transcript

Two genomic clones (HC3 and HC5) and a number of cDNA clones of human PVRs were obtained from genomic and complementary DNA libraries prepared from HeLa S3 cells by essentially the same method as that described by Mendelsohn *et al.* (1989). Nucleotide sequence analysis of a number of cDNA clones revealed the existence of at least four different forms of mRNAs with regard to their coding sequence. Possible translation products from these mRNAs were designated PVRα, PVRβ, PVRγ and PVRδ. The predicted amino acid sequences revealed that PVRα and PVRδ have the same structure as H20A and H20B, respectively, reported by Mendelsohn *et al.* (1989) except for one amino acid at position 67 (Thr ↔ Ala) which resulted from one nucleotide difference at position 199 (A ↔ G) (Figure 2). Clones which had exactly the same nucleotide sequence as that of H20A were also obtained from the cDNA library of HeLa S3 cells. Therefore, the nucleotide difference at position 199 may be due to an allelic difference. Coding sequences of mRNAs for PVRβ and PVRγ lack 135 and 159 nucleotides, respectively, including a sequence coding for a putative transmembrane domain indicated by a box in Figure 2.

To study the structure of the PVR gene, partial nucleotide sequence analysis of the receptor gene was carried out, and the nucleotide sequence was compared with those of the cDNA clones (Figures 1 and 2). The results indicate that the nucleotide sequence of PVRα mRNA was separated by seven introns. Exon 1 corresponds to sequences for the 5′ untranslated region and a putative signal peptide domain, exon 2 the first Ig-like domain, exon 3 the second Ig-like domain, exons 4 and 5 the third Ig-like domain, exon 6 a putative transmembrane domain, exon 7 a putative cytoplasmic domain, and exon 8 the carboxy terminus of the cytoplasmic domain and the 3′ untranslated region (Figures 1 and 2). This gene organization is characteristic of members of the Ig superfamily (Williams and Barclay, 1988). The results also support the predicted domain structure for the PVR protein. The precise transcriptional initiation site(s) is not determined yet.

Comparison of the nucleotide sequences also strongly suggests that the four mRNA species were generated by alternative splicing of a primary transcript from the PVR gene. PVRβ mRNA arises by splicing using a specific 5′ splice site at nucleotide position 1016 within exon 6 (Figure 2), and therefore lacks a part of the nucleotide sequence of exon 6 (Figures 1 and 2). PVRγ mRNA is generated when a whole nucleotide sequence of exon 6 is eliminated by joining exons 5 and 7 directly. PVRδ mRNA is generated when the 5′ splice site at exon 6 is not recognized as the splice site, resulting in an additional translated sequence of 26 nucleotides downstream of exon 6 (Figure 2). Consensus sequences for splice sites are observed in all cases but one where the dinucleotide GC and not GU is used at the 5′ splice site to generate PVRβ mRNA (Figure 2). These studies also revealed that the length of the PVR gene is ~20 kb (Figure 1).

Among 23 cDNA clones, 11 clones are for PVRα type, 10 clones for PVRβ type, one clone for PVRγ type and one clone for PVRδ type. This ratio of cDNA isolates may reflect a population of individual mRNA species in HeLa S3 cells. Thus, a considerable amount of PVRβ appears to be produced in HeLa S3 cells.

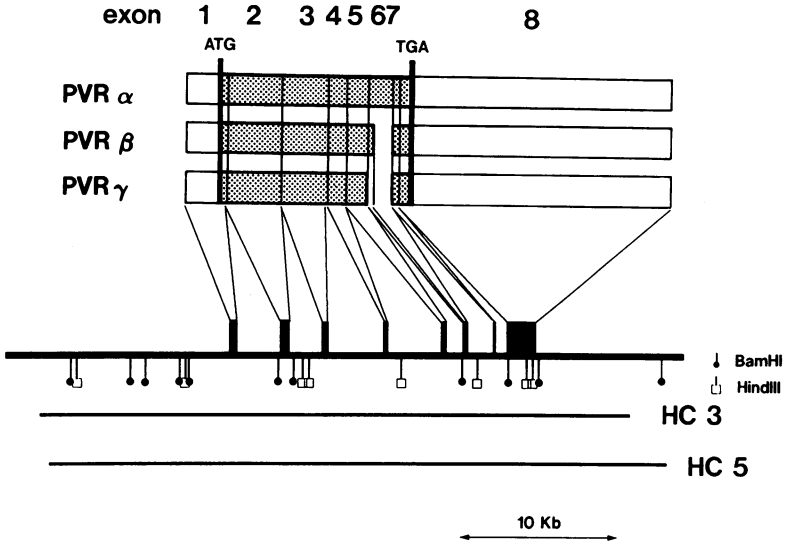

Fig. 1. Restriction map of the human PVR gene and multiple splicing of its transcript. Structures of mRNAs for PVRα, PVRβ and PVRγ are shown by open (untranslated sequences) and shaded (translated sequences) boxes. Numbers of exons are indicated at the top of the figure. Receptor regions are connected by thin lines to exons contained on a gene map shown underneath. Restriction cleavage sites of *Bam*HI and *Hin*dIII are indicated by vertical bars with closed circles and open boxes, respectively. Sequences of the human PVR gene in cosmids HC3 and HC5 are indicated by bars. A scale for length of nucleotides of the PVR gene is shown by a bar with arrowheads at both ends at the bottom of the figure. Precise initiation site(s) of transcription is not clear at present.

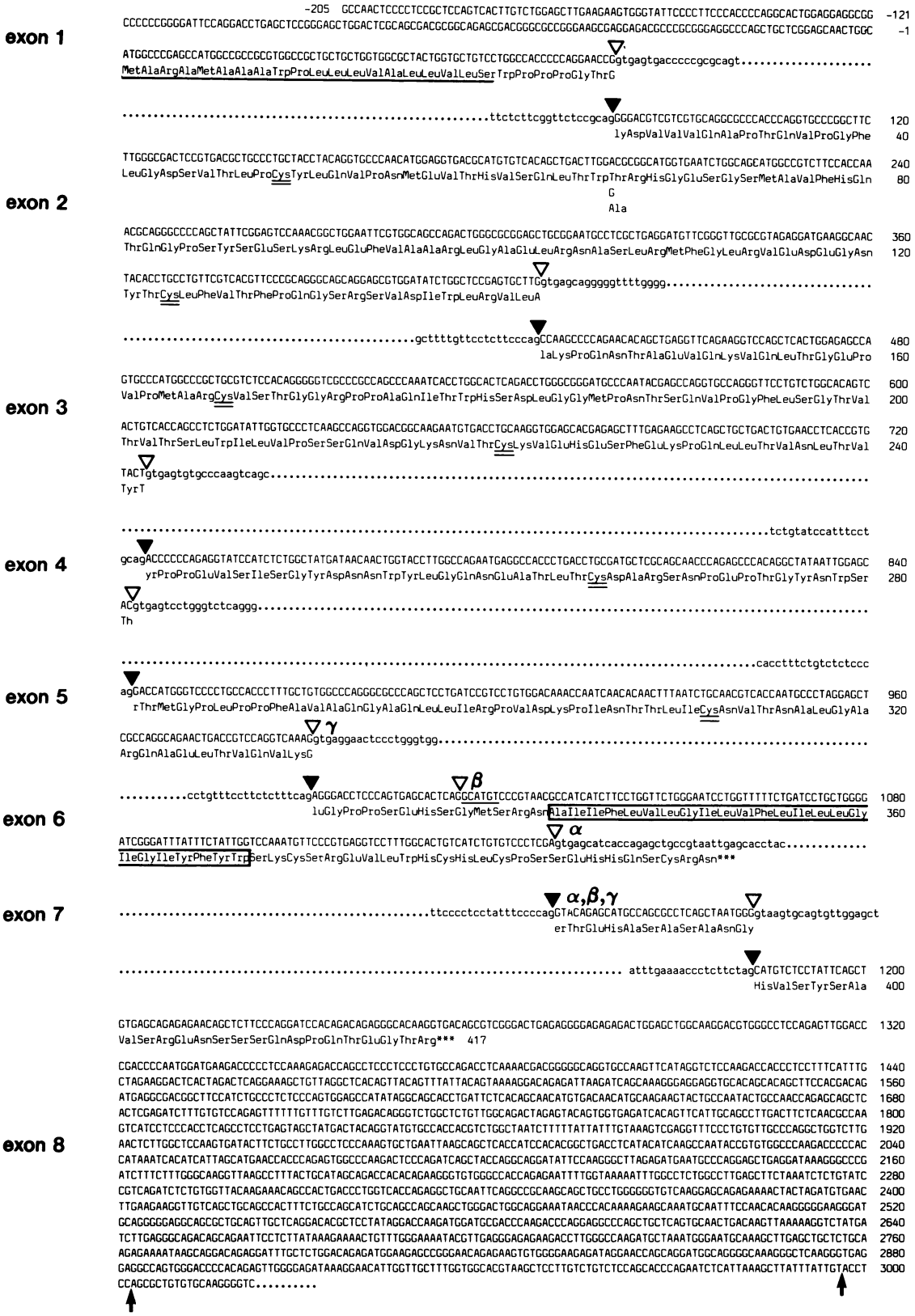

Fig. 2. Multiple splicing of an RNA transcript coding for PVR isoforms. Partial nucleotide sequence of the PVR gene is shown. Capital letters represent nucleotide sequences of exons for PVRα, and small letters those of introns for PVRα. Nucleotide positions beginning with the first in-frame ATG of PVRα cDNA are shown at the right margin. Donor and acceptor sites of splicing are indicated by $\triangledown$ and $\blacktriangledown$, respectively. Donor and acceptor sites which are specific for the generation of each isoform are indicated by $\triangledown\alpha$, $\triangledown\beta$, $\triangledown\gamma$ and $\blacktriangledown\alpha$, β, γ. Two different poly(A) sites are indicated by arrows. Amino acid sequences of PVRs are deduced from the nucleotide sequences of the cDNAs and amino acid positions are shown at the right margin. Putative signal peptide domain and transmembrane domain are indicated by a long underline and a box, respectively. Cys residues characteristic of Ig-like domains are indicated by double underlines. The guanine residue at position 199 in cDNA clones of H20A and H20B (Mendelsohn *et al.*, 1989) is replaced by an adenine residue in cDNA clones for PVRα, PVRβ, PVRγ and PVRδ. This nucleotide difference results in an amino acid change from Ala to Thr at position 67. The 5′ splice site that includes an unusual dinucleotide, GC, is indicated by a short underline.

Expression of PVR cDNAs in mouse L cells

Since PVRβ mRNA does not carry a nucleotide sequence encoding a putative transmembrane domain, it is possible that PVRβ molecules are released into the culture medium of the cells. To test this possibility, mouse cell transformants carrying cDNAs encoding PVRα and PVRβ were established as described in Materials and methods, and designated Lα and Lβ cells, respectively. In the transformants, these cDNAs are designed to be expressed under control of the SV40 promoter. The structures of the expression vectors used in this study are shown in Figure 3A. The receptor mRNAs expressed in the transformant cell lines were detected by Northern blot hybridization using an *Eco*RI–*Bam*HI cDNA fragment (nucleotide positions 278–1227) as a probe (Figure 3B). PVRα and PVRβ mRNAs of sizes expected from constructs of the expression vectors are observed on a blot (Figure 3B, lanes 2 and 3, respectively). PVR mRNAs of ~3.3 kb are detected in poly(A)$^+$ RNAs of HeLa S3 cells as reported by Mendelsohn *et al.* (1989) (Figure 3B, lane 1). Amounts of the mRNAs expressed in the transformants Lα and Lβ appeared to be >20-fold higher than that in HeLa S3 cells.

To determine whether PVRs are membrane-bound or secreted molecules, an indirect immunofluorescence study was performed using a monoclonal antibody P44 that blocked cellular receptors for poliovirus infection as described in Materials and methods (Figure 4). Bright peripheral fluorescence was visible on the surface of HeLa S3 cells (Figure 4A), and the mouse cell transformant Lα (Figure 4B) but not on the surface of Lβ (Figure 4C) or Ltk$^-$ cells (Figure 4D). Normal mouse serum used as a control did not show any fluorescence on any of the cell lines used here (data not shown). These results strongly suggest that PVRα molecules exist on the surface of the cells and that PVRβ molecules are a secreted isoform of PVR.

To confirm that PVRβ is released from the cells, immunoprecipitation of PVRs from culture fluids of the cells that were metabolically labeled with [^{35}S]methionine was carried out using a monoclonal antibody P44 as described

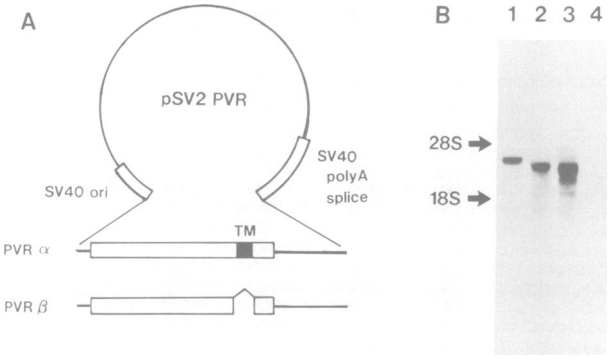

Fig. 3. Expression of PVR cDNAs in mouse cell transformants. Constructs of expression vectors pSV2PVRα and pSV2PVRβ are shown in (**A**). A β-globin cDNA in the expression vector pSV2-β-globin was replaced by cDNA for PVRα or PVRβ as described in Materials and methods. Results of Northern blot hybridization using an *Eco*RI–*Bam*HI cDNA fragment of PVRα cDNA as a probe are shown in (**B**). Poly(A)$^+$ RNA (10 μg) was prepared from HeLa S3 cells (lane 1) or Ltk$^-$ cells (lane 4) and used in this experiment, whereas 10 μg of total RNA was used in the case of Lα cells (lane 2) or Lβ cells (lane 3). Positions of 28S and 18S human rRNA are shown by arrows.

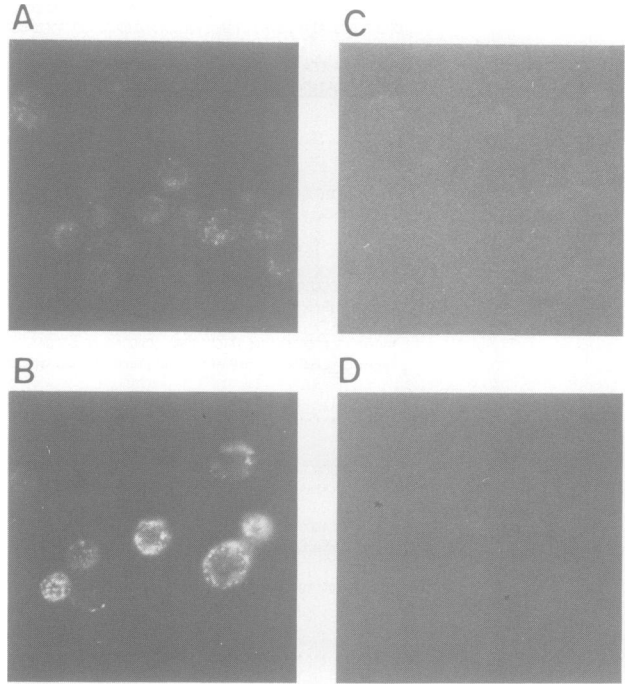

Fig. 4. Indirect immunofluorescence staining with monoclonal antibody P44. HeLa S3 cells (**A**), Lα cells (**B**), Lβ cells (**C**) and Ltk$^-$ cells (**D**) were incubated with mouse ascites fluid derived from P44. After removal of the ascites fluid, the cells were stained with a fluorescein-conjugated goat anti-mouse Ig as described in Materials and methods. Cells were observed with a fluorescence microscope.

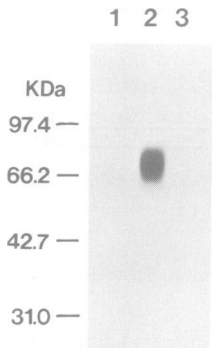

Fig. 5. Immunoprecipitation with monoclonal antibody P44. Lα cells (lane 1), Lβ cells (lane 2) and Ltk$^-$ cells (lane 3) were labeled with [^{35}S]methionine as described in Materials and methods. Materials that precipitated with monoclonal antibody P44 from the culture fluids were analyzed by 8% SDS–PAGE as described in Materials and methods. Positions of mol. wt markers are shown in kd on the left-hand side of the figure.

Table I. Production of poliovirus in mouse cell transformants

Cell	Log_{10} p.f.u./ml[a]		CPE
	1 h	12 h p.i.[b]	
HeLa S3	0	7.48	+
Lα	0	6.34	+
Lβ	0	4.70	–
Ltk$^-$	0	0	–

[a]Logarithmic value of plaque-forming units per ml.
[b]Post-infection.

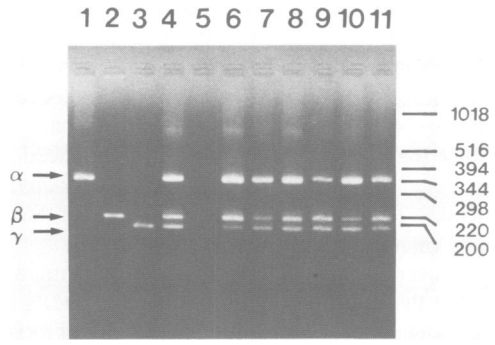

Fig. 6. PCRs performed on poly(A)$^+$ RNAs of cells and human tissues. PCRs were carried out on poly(A)$^+$ RNAs of Lα cells (lane 1), Lβ cells (lane 2), HeLa S3 cells (lane 4), Ltk$^-$ cells (lane 5), L_{HC5} cells (lane 6), human brain (lane 7), leukocytes (lane 8), liver (lane 9), lung (lane 10) and placenta (lane 11) as described in Materials and methods. PVRγ cDNA was used as a template for the PCR reaction and the products were applied on lane 3. The length of DNA fragments used as size markers are indicated in numbers of base pairs on the right-hand side of the figure. The positions of PCR products from mRNAs for PVRα, PVRβ and PVRγ are indicated by arrows with α, β and γ, respectively, at the left margin.

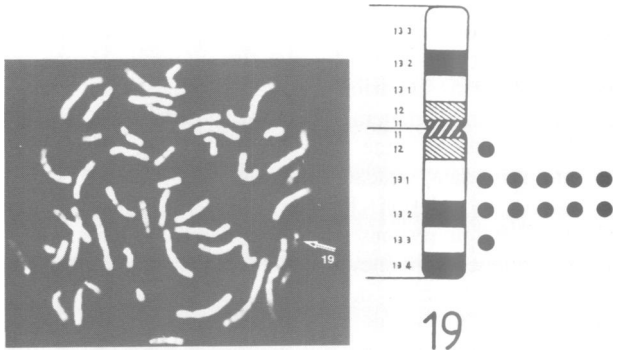

Fig. 7. *In situ* chromosome hybridization. An *in situ* hybridization experiment was performed as described in Materials and methods. Q-banded metaphase spread with a grain on human chromosome 19 is shown. Diagrammatic representation of chromosome 19 showing the clustering of grains on q13.1 → q13.2 is shown on the right-hand side of the figure.

in Materials and methods. The results are shown in Figure 5. A broad band at a position of apparent mol. wt 65−80 kd is specifically observed in immunoprecipitates from the culture fluid of Lβ cells (Figure 5, lane 2), but not those of Lα and Ltk$^-$ cells (lanes 1 and 3). Thus it appears that PVRβ is released into the culture medium. Since the mol. wt of PVRβ calculated from the deduced amino acid sequence is ~40 kd, it is likely that the material precipitated from the culture fluid is a glycosylated form of PVRβ. Indeed, nine possible *N*-linked glycosylation sites are observed in the amino acid sequence of PVRβ (Figure 2) (Mendelsohn *et al.*, 1989). Furthermore, H20A that is glycosylated *in vitro* using canine pancreatic microsomal membrane fraction migrates at a position of 67 kd on a polyacrylamide gel (E.Wimmer, personal communication). The broad band observed in Figure 5 may indicate the presence of a heterogeneity of post-translational modification of PVRβ. Alternatively, this may be due to partial degradation of PVRβ. In any event, these data indicate that PVRβ is a secreted isoform of PVR, and therefore the amino acid sequence missing in PVRβ has a function in anchoring the PVR molecule to the surface membrane of the cells.

Susceptibility of transformants to poliovirus infection

Since PVRβ appears not to stay on the surface of Lβ cells, it is very possible that the molecule is not a functional receptor for poliovirus infection. To investigate the function of PVRβ in virus infection, susceptibilities of the transformants Lα and Lβ to poliovirus infection were examined by measuring titers of virus produced in these cells at 12 h post-infection (p.i.) (Table I). To reduce the residual amount of poliovirus that causes an indefinite result, light-sensitive poliovirus was prepared and used for the infection as described in Materials and methods. As shown in Table I, infectious particles were produced both in Lα and Lβ cells as well as HeLa S3 cells, although the titer of virus produced in Lβ cells was much lower than that in Lα cells. Furthermore, no apparent CPE was observed on Lβ monolayer cells even if the cells were infected with virus at a multiplicity of infection (m.o.i.) of 100. These observations clearly indicate that only a very small population of the transformant Lβ is susceptible to poliovirus infection. The reason for this phenomenon is not known at present.

Poliovirus receptor mRNAs in human tissues

To determine whether the multiple splicing observed for PVR mRNAs in HeLa S3 cells also occurs in human tissues, poly(A)$^+$ RNAs of the brain, leukocytes, liver, lung and placenta of humans were analyzed by PCR as described in Materials and methods (Figure 6). Three bands of predicted sizes are observed in PCR products from poly(A)$^+$ RNA of all human tissues tested (Figure 6, lanes 7−11) as well as HeLa S3 cells (lane 4) and L_{HC5} cells (lane 6) that are mouse cell transformants with cosmid clone HC5 that carries a human PVR gene. No product is detected when RNAs from mouse Ltk$^-$ cells are employed (lane 5), nor in any cases of cells and tissues tested when a reverse transcription reaction is omitted (data not shown). The PCR products were cloned into a bacterial plasmid and the nucleotide sequences determined. The results clearly show that these products were synthesized from three mRNA isoforms of PVR (data not shown). Thus the data indicate that the multiple splicing generally occurs in human tissues and provides three different mRNA isoforms in respect of the nucleotide sequences encoding the transmembrane domain. PVRδ mRNA was not detected in this experiment because of the primers used for the polymerization reactions.

Chromosome mapping of the human PVR gene

In situ hybridization of ^{3}H-labeled *Eco*RI−*Bam*HI probe to human metaphase chromosomes was performed to identify the map position of the PVR gene as described in Materials and methods (Figure 7). Following *in situ* hybridization, 100 metaphases were analyzed. From a total of 110 grains on chromosomes, 12 (11%) were found over chromosome 19 with a significant clustering of silver grains on the long arm. Of these, 10 (83%) were found at the band q13.1 → q13.2 (Figure 7). The data indicate that the gene for the PVRs is located on this region of human chromosome 19. This result is compatible with the previous data reported by Miller *et al.* (1974) and Siddique *et al.* (1988).

Discussion

Two different functional cDNA clones (H20A and H20B) of human PVR mRNAs have been reported by Mendelsohn *et al.* (1989). Clones H20A and H20B are different in the nucleotide sequence encoding the putative cytoplasmic domain. We have cloned a number of cDNA clones of human PVR mRNAs. Sequence analysis of the cDNA clones revealed that two more mRNA isoforms encoding human PVRs, i.e. PVRβ and PVRγ, were generated in HeLa S3 cells and human tissues. Regions including the transmembrane domain are missing in both PVRβ and PVRγ molecules. PVRα and PVRδ appear to be identical molecules to the products of clones H20A and H20B, respectively, except for one nucleotide at position 199 that may represent an allelic difference. Thus, four different mRNA isoforms have so far been discovered for human PVRs. We demonstrated in this study that these four mRNAs are possibly derived by alternative splicing mechanisms from a single gene located at the band q13.1 → q13.2 of human chromosome 19. It is of interest that one of the 5′ splice sites for generating PVRβ is an unusual dinucleotide, GC, which has been observed only in a few cases (Padgett *et al.*, 1986).

The cDNA for PVRβ does not contain the sequence encoding the putative transmembrane domain. Indeed, PVRβ is released into the culture fluid of Lβ cells. Since Lβ monolayer cells did not display apparent CPE upon infection with poliovirus, PVRβ seemed not to be a functional PVR. However, Lβ cells showed susceptibility, albeit very low, to poliovirus infection. It is unlikely that poliovirus infects the cells without recourse to its receptor on the cell surface, since Ltk$^-$ cells are totally resistant to infection. Thus, the mechanism for this phenomenon is not known at present. It is possible, however, that Lβ cells are permissive for poliovirus infection during very short periods when PVRβ is transiently on the surface membrane. The function of PVRγ as cellular receptor for poliovirus infection was not examined in this study. However, it seems plausible that PVRγ is also a soluble form of PVR and is therefore not a functional receptor, since the amino acid sequence of the transmembrane domain encoded by exon 6 is missing in PVRγ as well.

PVR mRNAs have been detected in every human tissue tested by Northern blot hybridization (Mendelsohn *et al.*, 1989). Since this technique cannot discriminate between any two PVR mRNA isoforms expressed in tissues, we employed the PCR technique to detect individual mRNA isoforms in human tissues. The results indicated that three mRNA isoforms for PVRα, PVRβ and PVRγ existed in every poly(A)$^+$ RNA preparation from human tissues tested (Figure 5; note that the primers chosen did not permit detection of PVRδ). However, it is still possible that expression of individual forms of PVR is different in cell types in which alternative splicing specific to the cell types occurs. This may in turn regulate the permissivity of the cells for poliovirus infection. Indeed, indirect immunohistochemical study on the human brainstem using a monoclonal antibody against PVRs suggested that only limited types of cells express the receptor molecules (Shepley *et al.*, 1988) that were probably PVRα type and PVRδ type. Thus, elucidation of post-transcriptional regulation including splicing as well as transcriptional regulation specific to cell types is essential to understand the molecular basis of the pathogenesis of poliovirus. It is of interest to know whether binding of poliovirus to soluble PVR molecules leads to inactivation of the virion. The spread of the virus through the body could be significantly influenced by the occurrence of soluble receptor molecules. Indeed, preliminary results indicate that the soluble PVR molecules produced in the baculovirus vector system are able to bind and inactivate the virus (S.Koike, T.Yoneyama, I.Ise, T.Horiuchi, Y.Saeki and A.Nomoto, manuscript in preparation).

The predicted amino acid sequence of human PVR revealed that the molecule was a member of the Ig superfamily (Mendelsohn *et al.*, 1989). This family of molecules is undoubtedly one of the important groups not only in immunity but also in the mediation of cell surface recognition for the control of cell behavior in various tissues (Williams and Barclay, 1988). There are a number of examples of cell surface proteins that have soluble counterparts, including other members of the Ig superfamily such as IgM (Early *et al.*, 1980) and N-CAM (Gower *et al.*, 1988). Different forms of these molecules also arise by alternative splicing, which depends on a stage of development of cells or tissues. Although the biological function(s) of the PVRs is totally unknown at present, the discovery of secreted forms of the molecules may give an insight into the role of PVRs. PVRs may function not only as receptors but also as ligands in the human body.

It is almost impossible to investigate specific expression of the PVR gene in various cells and tissues of humans and monkeys during development. Accordingly we recently made transgenic mice carrying a human PVR gene. Preliminary data indicate that the mice are sensitive to poliovirus inoculation. Furthermore, the transgenic mice showed different responses to poliovirus strains with different neurovirulence. This new experimental animal model must be a powerful tool to investigate the relationship between poliovirus replication and the receptor, the regulation of PVR gene expression and the physiological function of PVR molecules. The mouse model may also be useful for tests to assess the quality of oral live poliovirus vaccines.

Materials and methods

DNA clones of human PVRs

A cosmid library of HeLa S3 cell DNA was prepared essentially as described by Maniatis *et al.* (1982) using DNAs partially digested with *Sau*3A1. Plasmid pTL5 (Lund *et al.*, 1982) and *Escherichia coli* ED8767 were used as a vector and its host, respectively.

For the preparation of a cDNA library, total RNA of HeLa S3 cells was isolated as described in Okayama *et al.* (1987). Poly(A)$^+$ RNAs were then selected by oligo(dT)–cellulose column chromatography (Aviv and Leder, 1972). Complementary DNAs were synthesized by the method of Gubler and Hoffman (1983) using either oligo(dT)$_{12-18}$ or random 6mer as primers. Double-stranded cDNAs were inserted into the *Eco*RI site of vector λgt10 as described by Watson and Jackson (1985). Ligated DNA was packaged *in vitro* and plated onto *E.coli* C600hflA150.

To isolate DNA clones of PVRs from the genomic and complementary DNA libraries of HeLa S3 cells, probe DNAs were prepared essentially as described by Mendelsohn *et al.* (1986, 1989). Briefly, genomic DNA of the secondary mouse cell transformants sensitive to poliovirus was prepared, partially digested with *Sau*3A1, and then fractionated by sucrose density gradient centrifugation. DNA fragments in a range of 15–20 kb in length were ligated to the *Bam*HI site of vector EMBL3 and packaged *in vitro*. Phages were plated onto *E.coli* P2392. Genomic clones that hybridized with human *Alu* repeat Blur-8 were selected and used as probes for the isolation of genomic and complementary DNA clones of human PVRs from the libraries prepared as above. As a result, two genomic clones that give the permissivity for poliovirus infection to mouse Ltk$^-$ cells were isolated and designated HC3 and HC5. Restriction mapping of the clones

revealed that they have a long overlapping nucleotide sequence (see Figure 1). Similarly, a number of cDNA clones were isolated from the cDNA library of HeLa S3 cells.

DNA sequencing

Cloned DNAs were subcloned into the appropriate sites of pUC118 and pUC119, and the nucleotide sequences were determined by the dideoxy termination method (Sanger *et al.*, 1977) using Sequenase sequencing kits (United States Biochemical Corporation). In some cases, synthetic oligonucleotides corresponding to appropriate sites of cloned DNAs were used as sequencing primers.

Cells and viruses

HeLa S3 cells were propagated as suspension culture in RPMI media 1640 supplemented with 5% newborn calf serum (NCS). African green monkey kidney (AGMK) cells were cultured in Dulbecco's modified minimum essential medium (DMEM) supplemented with 5% NCS. Mouse Ltk^- cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). Mouse cell transformants (Ltk^+) were usually maintained in DMEM containing 10% FCS, 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine (HAT medium).

Poliovirus type 1 Mahoney strain was grown in suspension cultured HeLa S3 cells at 37°C. The virus stocks were prepared by freeze–thawing of the infected cell cultures three times followed by centrifugation to remove cell debris. Virus titers were measured by conventional plaque assays using AGMK monolayer cells (Kawamura *et al.*, 1989).

Monoclonal antibody against PVRs

Monoclonal antibody P44 which blocks cellular receptors for poliovirus was prepared according to the method described by Minor *et al.* (1984). The subtype of the antibody was determined to be IgG2b by using a Mouse Monoclonal Antibody Isotyping Kit (Amersham, UK). Ascites fluid after growing the hybridoma cells in Pristan-primed BALB/c mice was used as the antibody in this study.

Expression vectors

The *Eco*RI site of the corresponding 5′ end of the cDNA clone for PVRβ was filled in using the Klenow fragment and ligated with *Hin*dIII linkers. A *Hin*dIII–*Bgl*II cDNA fragment carrying sequence for the whole translated region was inserted into the *Hin*dIII–*Bgl*II site of a plasmid vector pSV2-β-globin (Gorman, 1985) so that the cDNA was designed to be expessed under the control of the SV40 early promoter. This plasmid was designated pSV2PVRβ. A similar plasmid, designated pSV2PVRα, containing a sequence for PVRα was constructed by replacing the *Kpn*I–*Bgl*II fragment of pSV2PVRβ by the corresponding sequence of PVRα cDNA.

Transfection

Mouse Ltk^- cells in 60 mm plastic dishes were transfected with 5 μg of pSV2PVRα, pSV2PVRβ or cosmid DNA together with 0.25 μg of plasmid pTK carrying the herpes simplex virus thymidine kinase gene by the calcium phosphate co-precipitation method (Chen and Okayama, 1988). After 16 h transfection, the cells were rinsed, refed with growth medium and incubated at 37°C for 24 h. The cells were then passaged with 5-fold dilution and cultured for another 24 h. Transformants (tk^+) were selected in HAT medium. Cell lines were established by two cycles of limiting dilution.

Northern blot hybridization

Northern filters were prepared as described by Thomas (1980) except that the transfer was carried out by using Vacugene transfer apparatus (LKB). An *Eco*RI–*Bam*HI cDNA fragment (nucleotide positions 278–1227) was labeled with ^{32}P by nick translation (Rigby *et al.*, 1977) and used as a probe. Hybridization was performed in 1 M NaCl, 50 mM Tris–HCl, pH 7.5, 50 mM EDTA, 0.1% Sarkosyl, 0.2% bovine serum albumin, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone at 65°C overnight. Filters were washed twice with 0.1 × SSC, 0.1% SDS at 65°C for 30 min.

Immunofluorescence

HeLa S3, Lα, Lβ and Ltk^- cells were incubated with mouse ascites fluid derived from P44 (1:100 dilution in DMEM containing 5% FCS) at 0°C for 1 h, and then washed three times with the ice-cold medium. After removal of the medium, the cells were incubated at 0°C for 1 h in the dark with a fluorescein-conjugated goat anti-mouse Ig (1:100 dilution in DMEM containing 5% FCS) (Cappel). The cells were observed with a fluorescence microscope (Zeiss Standard).

Immunoprecipitation

Lα, Lβ or Ltk^- cells (5×10^5 cells) were metabolically labeled with [^{35}S]methionine (Amersham, 100 μCi/ml) in methionine-free medium overnight. 1 ml portions of culture fluids were incubated with 5 μl of normal mouse serum at 4°C for 1 h. To the mixture, 100 μl of protein A–Sepharose (Pharmacia) [50% suspension in phosphate-buffered saline (PBS)] was added and incubated for another 1 h with gentle shaking. After the centrifugation to remove materials reacted with normal mouse serum, 5 μg of monoclonal antibody P44 was added to the supernatants and the mixture was kept at 4°C for 1 h. Protein A–Sepharose was then added to the mixture and incubated for 1 h as above. Resins were washed 10 times with 1 ml of ice-cold PBS. Radioactive materials were eluted from the resins with 1% SDS and analyzed by 8% SDS–PAGE in Laemmli's buffer system (Laemmli, 1970).

Sensitivity of mouse transformants to poliovirus

Poliovirus which can be inactivated by light was prepared according to the method described by Madshus *et al.* (1984). Briefly, HeLa S3 monolayer cells were infected with poliovirus type 1 Mahoney virus at a m.o.i. of 0.01 in the presence of 10 μg/ml neutral red. The progeny virus produced was passaged again under the same conditions except that the m.o.i. was ~1. Virus preparations were performed in the dark. The virus was tested for sensitivity to light. The results showed that ~100 out of 10^8 plaque-forming units (p.f.u.) of the virus remained after illuminating the virus solution with a 60 W bulb at a distance of 70 cm for 30 min.

HeLa S3, Lα, Lβ and Ltk^- cells (10^5 cells/dish) were infected with poliovirus at a m.o.i. of 10, and incubated at 37°C in the dark. At 1 and 12 h after infection, virus was harvested by freeze–thawing of the infected cultures three times followed by centrifugation to remove cell debris. The virus preparations were exposed to light, and the titers of virus were measured as described above. Evidence of virus-induced CPE was examined microscopically at 12 h p.i.

Polymerase chain reaction

Three steps of PCR were performed using a DNA thermal cycler (Perkin Elmer Cetus). Complementary DNAs to poly(A)$^+$ RNAs (1 μg) were synthesized using avian myeloblastosis virus reverse transcriptase (Seikagaku Kogyo Co.) and 1 μg of primer no. 24 (corresponding to nucleotide positions 1255–1274) in a 20 μl reaction mixture. After the reverse transcription, a 10 μl portion of the reaction mixture was mixed with 0.5 μg of primer no. 25 (positions 881–900). The first PCR was performed in 100 μl of reaction mixture for 30 cycles by denaturing at 94°C for 1 min, annealing at 55°C for 2 min, and extension reaction at 72°C for 2 min with GeneAmp™ (DNA Amplification Reagent kit, Perkin Elmer Cetus). The second step of PCR was carried out using 2 μl of reaction mixture of the first step PCR and 1 μg each of primers no. 12 (positions 1234–1253) and no. 14 (positions 901–920). Other conditions were the same as those in the first step PCR. The reaction mixture of the third step PCR contained 5 μl of the mixture of the second step PCR and 1 μg each of primers no. 12 and no. 14. After three cycles of the third step PCR products were analyzed by electrophoresis on a gel containing 3% NuSieve™ GTG agarose (FMC BioProducts) and 1% agarose. Poly(A)$^+$ RNAs of human tissues were purchased from CLONTECH Laboratories, Inc.

In situ hybridization

The procedures for *in situ* hybridization to metaphase chromosomes were essentially the same as those described by Yoshida *et al.* (1986). Briefly, an *Eco*RI–*Bam*HI cDNA fragment (nucleotide positions 278–1227) of PVR cDNA was labeled with ^3H by nick translation or random priming and used as a probe for hybridization to chromosomes of normal male lymphocytes. After the hybridization, slides were washed, dehydrated and exposed to nuclear track emulsion (Sakura NR-M2, Konishiroku, Tokyo) for 10 days. Q-banded chromosomes (Yoshida *et al.*, 1975) were analyzed directly under the microscope for the presence of silver grains overlying or close to the chromatids.

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