The Promoters for Human and Monkey Poliovirus Receptors

**REQUIREMENTS FOR BASIC AND CELL TYPE-SPECIFIC ACTIVITY**

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The cellular receptors for poliovirus (PVR) are glycoproteins belonging to the immunoglobulin superfamily. Functional receptors for poliovirus are only expressed by primates; known rodent homologues lack the ability to bind virus due to amino acid differences. Human poliovirus infections are targeted to the gastrointestinal tract and, rarely, to motor neurons in the central nervous system. Available evidence suggests that poliovirus uses only one cellular receptor, implying that the tissue tropism of poliovirus is likely to be related to the expression of the human PVR (hPVR). However, low levels of expression of hPVR-specific mRNAs can be detected in many human tissues other than the apparent target cells. The nonpathogenic function of hPVR is unknown. For a study of the transcriptional control of hPVR expression, we have isolated and characterized the promoter of the hPVR gene. Deletion analysis defined an approximately 280 base pair minimal promoter fragment that: 1) lacks TATA- and CAAT-like elements, 2) is distinguished by a high GC content, and 3) promotes transcription at multiple start sites. The pattern of activity caused by transfection of serial 5′- and 3′-promoter deletions is almost identical in HEp2, HeLa, COS-1, and mouse L929 cells, indicating a similar transcriptional regulation of the hPVR promoter in these cell lines. However, on transfection of Raji cells, a Burkitt's lymphoma cell line harboring a transcriptionally inactive hPVR gene, all promoter reporter constructs tested exerted only residual activity. These results suggest that the cis-element(s) governing cell type-specific hPVR expression resides in the minimal promoter region. We also report the sequences of the promoters of two monkey homologues to hPVR (AGMa1 and AGMa2). Transcripts encoding the monkey poliovirus receptors originate from a region analogous to that identified for hPVR transcripts.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X94226 for hPVR, X94227 for AGMa1, and X94228 for AGMa2.

†The abbreviations used are: hPVR, human poliovirus receptor; mPVR, monkey poliovirus receptor; AGM, African green monkey; bp, base pair; kb, kilobase pair; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; CAT, chloramphenicol acetyltransferase; NTR, nontranslated region.

The human receptors for poliovirus (hPVR)† are cell surface proteins possessing three immunoglobulin-like domains, designated V-C-C (1, 2; reviewed in Ref. 3). Analyses of cDNAs and of genomic DNA suggested that hPVR-related proteins are expressed in the forms of four splice variants: two membrane-bound (hPVRα and hPVRβ) and two secreted (hPVRδ and hPVRγ) polypeptides (Refs. 1 and 2 and Fig. 1). The membrane-bound receptors hPVRα and hPVRβ differ only in the sequences of their cytoplasmic C-terminal domains, and they are highly glycosylated (4, 5). Moreover, receptor hPVRα is phosphorylated on one or more serine residues of its cytoplasmic domain (6). The nature of the secreted splice variants (hPVRδ and hPVRγ) has been largely inferred only from analyses of their corresponding mRNA species (1).

Humans are the only natural hosts for poliovirus. Monkeys, however, can be experimentally infected, because they express receptors homologous to hPVR. Koike et al. (7) have shown that, in contrast to hPVR, monkey poliovirus receptors (referred to in the following as mPVRs) of African green monkey cells are encoded by two related genes AGMa1 and AGMa2 (African green monkey receptor). The predicted gene products mPVRa1 and mPVRa1 (for AGMa1) and mPVRa2 (for AGMa2) are integral membrane glycoproteins that were shown to be functional poliovirus receptors (7). Secreted splice variants of AGMa1 and AGMa2 have not been detected (7).

A gene (MPH, mouse poliovirus receptor homologue) homologous to the human and monkey poliovirus receptor genes exists also in mouse cells (8, 9). Similarly to hPVRs and mPVRs, the receptor homologues encoded by MPH belong to the immunoglobulin superfamily possessing the putative domain structure V-C-C (reviewed in Ref. 3). The MPH gene product, however, is unable to function as a poliovirus receptor due to differences in its amino acid sequence in comparison with the human and monkey receptors. This is true particularly in the case of the V domain, the structure of hPVR that has been shown to bind the virus (9–12).

Recently, two new human genes, PRR1 and PRR2 (poliovirus receptor-related genes) have been deduced from cDNA sequences that can be predicted to encode proteins related to hPVR (13, 14). Therefore, it appears that a gene family encoding PVR-like molecules may exist in humans similar to families of Ig-like CEA (15) and PSG genes (16). Interestingly, the latter two gene families map to human chromosome 19q, as does the hPVR gene (1, 17–19).
The cellular function(s) of hPVR and the hPVR-related proteins in humans, monkeys, and mice is obscure. Early studies have led to the prediction that the observed narrow tissue tropism of poliovirus would mirror the distribution of hPVR expression in human tissues (20). However, results of Northern blot analyses have indicated that transcripts related to hPVR sequences can be detected in nearly all human tissues that have been analyzed (1, 2; see discussions in Refs. 21 and 22).

Since human monocytes express hPVR (23), the apparent ubiquity of hPVR transcripts in human tissues may be explained, at least in part, by contaminating blood cells in tissue samples. It is also possible that the probes used to detect hPVR-related transcripts cross-reacted with transcripts of PRR1 and PRR2.

In our efforts to characterize cellular function(s) for hPVR and to gain insight into the pathology of poliovirus infection in humans, we have isolated and characterized the promoters of the hPVR and AGM genes. Structural as well as functional analyses indicate that the expression of the hPVR gene is controlled by a TATA and CAAT box-deficient promoter. Our results suggest that cis-acting elements of the hPVR core promoter and trans-acting factors available in cell lines of primates and mouse origin generally support hPVR expression, a result in concurrence with the expression of the hPVR gene in transgenic mice (24, 25). However, the fine tuning of promoter activity of the hPVR gene may differ slightly depending on the cell type investigated. Interestingly, hPVR is not expressed in a variety of hematopoetic cell lines, including the Burkitt's lymphoma cell line Raji. We provide evidence that the hPVR core promoter is inactive in these cells, indicating that this sequence harbors information for the control of basal and cell type-specific expression of the hPVR gene.

MATERIALS AND METHODS
Isolation of hPVR Genomic DNA
A chromosome 19-specific genomic library was purchased from the American Type Culture Collection (ATCC 57766; library name LL19NL01). The bacterial strain used was LE392 (ATCC 33572).

Screening the Genomic Library—2.5 × 10^6 plaque-forming units were screened with a 32P-labeled Bss HII-SacI fragment (153 bp) isolated from the 5′-NTR of cDNA clone H20A (2). Positive plaques were picked, and 1500 plaque-forming units each were plated for a second screen, resulting in the identification of single positive plaques.

Growth of Plasmid DNA and DNA Preparation—Competent L392 bacteria were infected with 10^7 plaque-forming units of λ-phage. These suspensions (70 ml) were incubated at 37°C until lysis of the bacteria was essentially the same, but 2×10^7 cells, and the viscous solution was homogenized by pipetting. The lysate was loaded onto a cushion of 5.7 M CaCl_2 and spun for 18 h at 36,000 rpm in an SW 41 rotor. The RNA pellet was dissolved in 500 μl of buffer (10 mM EDTA, pH 7.0, 0.5% N-lauroylsarcosine, 5% 2-mercaptoethanol, 5% phenol) and precipitated twice with ethanol.

Northern Blotting—Fifty μg of total RNA were dissolved in 50 μl of denaturing buffer (2.2 M formaldehyde, 50% formamide, 10% 10× electrophoresis buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0)), heated to 65°C for 15 min, mixed with 5 μl of 10× FF (10% Ficoll 400, 0.2% bromphenol blue), electrophoresed through a 1.2% agarose gel (containing 2.2 M formaldehyde), and blotted onto nitrocellulose.

For hybridization the filter was incubated overnight with hybridization buffer (50% formamide, 50 mM Pipes, pH 6.5, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% Denhardt’s solution, 400 μg/ml yeast tRNA) at 42°C before radiolabeled probe was added. Incubation at 42°C was continued for another 24 h. The filter then washed with 2× SSC, 0.1% SDS twice for 20 min at room temperature followed by two additional washes with 0.1× SSC, 0.1% SDS for 20 min each. The filter was exposed to an x-ray film using an intensifying screen.

Radioactive Labeling
For DNA labeling the Life Technologies, Inc. random-primed DNA labeling kit and [32P]dCTP (Amersham Corp.) were used according to the instructions of the manufacturers.

End labeling of oligodeoxynucleotides was done by incubating 5 pmol of an oligonucleotide in a total volume of 25 μl with 50 μCi of γ-32PdATP (Amersham) and 3 units of polynucleotide kinase (Biolabs) for 15 min at 37°C. The labeled samples were desalted using a Sephadex G25 column (Pharmacia).

PCR
PCR was done using a Perkin-Elmer or Biometra thermocycler. Conditions for the generation of deletion fragments were: 10 ng of plasmid template, 50 pmol of each primer, 5 μl of 10× buffer, 1 μl of 10 mM dNTP mix, 5 μl of formamide, and 0.5 μl of Toy polymerase (2.5 units, Boehringer Mannheim) in a total reaction volume of 50 μl. PCR was started with five cycles of 1 min at 94°C, 30 s at 37°C, and 1 min at 72°C, followed by 25 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C. For genomic DNA (100 ng) as template the probe composition was essentially the same, but 2 μl of 10 mM dNTPs and 1 unit of vent polymerase (Biolabs) were used. PCR was done for 35 cycles. For cloning the PCR products were cut with appropriate restriction enzymes and gel purified. The oligonucleotides used were (restriction sites are underlined): S20, 5′-GGCCCGGGGAGACAGCGTC-3′; S22, 5′-CTCTTAGATGACTGTACGGACG-3′; 1729, 5′-CCAGAATTCACGCGG-3′; 3745, 5′-GGCCCTGAGGAGACAGCGTCCAT-3′; 3912, 5′-GAGACCCGCGGCGGCTCAG-3′; 4141, 5′-GGAGATCTCGGGCCGTCAG-3′; 4411, 5′-GGAGATCTCGGGCCGTCAG-3′; 4438, 5′-GGGCTGACGCTGAGCAGGAC-3′; 4453, 5′-GGGCTGACGCTGAGCAGGAC-3′; 4526, 5′-GGGCTGACGCTGAGCAGGAC-3′; 4527, 5′-GGGCTGACGCTGAGCAGGAC-3′; 4529, 5′-GGGCTGACGCTGAGCAGGAC-3′; 4531, 5′-GGGCTGACGCTGAGCAGGAC-3′.

Primer pairs used to generate the promoter and promoter deletion constructs were: 4438 and 4410 (F), 4438 and 4526 (G20), 4438 and 4526 (G17), 4532 and 4529 (BE), 4538 and 4529 (AGMa2), and 4538 and 4513 (AGMa1).
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RACE

Rapid amplification of cDNA ends (5′-RACE) was done according to the protocol described by Frohman (26) with the following modifications.

Reverse Transcription (RT)—Partially purified mRNA from HEp2 and JA-1 cells was prepared using a Quickprep mRNA purification kit (Pharmacia). One μg of mRNA in 2 μl of water containing 50 mmol of primer transferred 4463 from 1729, see below) was heated to 80 °C and then allowed to cool to 40 °C. After a 2-h incubation period at 40 °C, buffer, dithiothreitol, dNTPs, RNAsin, and Superscript reverse transcriptase were added according to the manufacturer’s instructions (Life Technologies). The reaction mixture was incubated at 40 °C for 90 min.

First PCR—GS-P1 was primer 1729. Five cycles with 30 s at 94 °C, 3 min at 48 °C, and 45 s at 72 °C were done, followed by 30 cycles with 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C. The annealing temperature was increased by 0.3 °C/cycle.

Second PCR—GS-P2 was primer 4529. Thirty cycles with 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C were done. The increment for the annealing temperature was 0.2 °C/cycle.

RACE products were cut with BglII-HinDIII and cloned into Bluescript KS− and BamHI-HindIII, and colonies were sequenced with T3 primer.

Cloning of Reporter Plasmids

The promoter fragments generated by PCR (see above) were cloned into the Nhel-BglII site of the pGL2-Basic vector (Promega), and the vector and insert transitions were sequenced. The H reporter construct was cut with SacI, and the insert was replaced by the 2.9-kbp SacI fragment of p5.7/7, thus generating the large promoter/reporter vector (promoter insert of approximately 3000 bp).

Cell Culture

HeLa R19, HEp2 (a human laryngeal cell line), Hep G2 (a human liver cell line), COS-7, COS-1 (African green monkey kidney cell lines), SK-N-SH (a human neuroblastoma-derived cell line), 293 (a human kidney cell line), RD (a human rhabdomyosarcoma cell line), and mouse L cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 8% fetal bovine serum. JA-1 cells (a mouse L cell line) cells as well as all other BL cell lines (F126, JBL2, JI, LY66, LY67, LY91, BL40, BL60, BL70, BL72, BL90, and BL99) were grown in RPMI 1640 medium with 8% fetal bovine serum. L735, CEM (human T leukemia cell lines), HL60, RD (a human rhabdomyosarcoma cell line), and mouse SK-N-SH (a human neuroblastoma-derived cell line), 293 (a human liver cell line), COS-7, COS-1 (African green monkey kidney cell lines), and mouse L cell line harboring the p5.7/7 fragment of p5.7/7, thus generating the large promoter/reporter vector (promoter insert of approximately 3000 bp).

Cell Culture

HeLa R19, HEp2, Hep G2, HeLa, and L cells were transfected by the calcium phosphate procedure. 5–20 μg of test plasmid, 2 μg of pSV2CAT (in combination with luciferase vectors only), and 62.5 μl of 2 m CaCl2 in a total volume of 500 μl were combined dropwise with 500 μl of ice-cold 2 × Hank’s balanced salt solution. The precipitate was added to the cells. Four hours after the medium was removed, and a solution of 20% glycerol in Hank’s balanced salt solution was added. Following a 2-h incubation period at 40 °C, buffer, dithiothreitol, dNTPs, RNAsin, and Superscript reverse transcriptase were added according to the manufacturer’s instructions (Life Technologies). The reaction mixture was incubated at 40 °C for 90 min.

First PCR—GS-P1 was primer 1729. Five cycles with 30 s at 94 °C, 3 min at 48 °C, and 45 s at 72 °C were done, followed by 30 cycles with 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C. The annealing temperature was increased by 0.3 °C/cycle.

Second PCR—GS-P2 was primer 4529. Thirty cycles with 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C were done. The increment for the annealing temperature was 0.2 °C/cycle.

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Raji cells were electroporated. 107 cells/transfection were spun, and the cell pellet was resuspended in medium without serum. The suspension was combined with 5–20 μg of test plasmid and 2 μg of pSV2CAT. Electroporation was done at 340 V and 1180 microfarads with a Life Technologies electroporator (electrode gap, 0.4 cm). Cells were allowed to sit for 10 min at room temperature and were then transferred with fresh medium and serum into culture flasks.

All transfected cells were harvested 36 h after transfection, and cell extracts (usually 400 μl) were made using the reporter lysis buffer from Promega.

CAT activity was measured with a CAT enzyme-linked immunosorbent assay kit (Boehringer Mannheim), whereas luciferase activity was determined using the luciferase assay system from Promega.

RESULTS

Cloning of the 5′-Region of the hPVR Gene—The hPVR gene maps to chromosomes 19 (19q13.1–13.2; Refs. 1 and 17–19). Using cDNA sequences and cosmid clones, Koike et al. (1) have determined the intron and exon structure of the hPVR gene (Figs. 1A and 2A). They found that the first coding exon hybridized to a 5.7-kbp genomic BamHI fragment. However, neither the start site of transcription nor the promoter of the gene has been characterized.

To isolate the putative promoter region, a human chromosome 19-specific genomic library was screened for λ-phages harboring the 5′-region of the hPVR gene (see “Materials and Methods”). The 5′-NTR of cDNA clone H20B maps to the center of the 5.7-kbp BamHI fragment (Fig. 2B, open box; H20B contains the most extended 5′-sequence information). The BamHI-SacI fragment was likely to contain part or all of the promoter of the hPVR gene, for which reason it was subcloned and its nucleotide sequence was determined. Appropriately, this BamHI-SacI fragment was found to represent the 5′-end of a cosmid insert containing a functional hPVR gene (not shown).

This cosmid has been used previously by us to generate the mouse cell line 4A-1 expressing functional hPVRα and hPVRβ isotypes (4). The BamHI-SacI fragment was cloned into a CAT reporter plasmid and found to exert promoter activity.2 As discussed in greater detail below, the promoter region lacked TATA- and CAAT-like elements.

hPVR-specific mRNAs—Northern blot analyses were carried out to determine the level of transcriptional expression and to estimate the size of hPVR mRNAs in various cell lines. As can be seen in Fig. 3, hybridization of RNAs of different human and monkey cell lines (HEp 2, HeLa, RD, SK-N-SH, and COS7) with a probe recognizing all splice variants of the hPVR gene yielded a single band in all cases. A similar signal was obtained with RNAs of the human cell lines HL60 and 293, whereas, interestingly, RNAs of a variety of hematopoietic cell lines (CEM, L735, F126, 549, JI, LY66, LY67, LY91, BL40, BL60, BL64, BL70, BL72, BL90, and BL99) gave no detectable signals in Northern blots (data not shown). The intensity of the hPVR signal from cells known to be permissive for poliovirus replication suggests rather low levels of hPVR-related mRNA. In contrast to the blots using human and monkey cell line RNAs, experiments carried out using RNA obtained from JA-1 cells yielded a very intense signal, showing some bands below and above the authentic hPVR band. JA-1 cells are mouse cells stably transformed with the hPVR gene (see above); they harbor approximately 100 copies of the hPVR gene per cell. This is

S. Schwarz, unpublished results.
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Methods.

For details see “Materials and Methods.” The distribution of hPVR exons is given schematically in roman numerals. Fine mapping of the upstream half of the 5.7-kbp BamHI fragment harboring the hPVR promoter. The BamHI-SacI fragment was sequenced and the SmaI site (2.88 kbp) and the SacI (2.9 kbp) map within the 5′-portion of cDNA clones H20A and H20B (2). Shaded boxes, Alu-repetitive elements. Open box, 5′-part of cDNA clone H20B. The XhoI site determining the 5′-end of the hPVR promoter sequence in Fig. 4 is given in bold letters.

Fig. 2. Restriction maps of hPVR genomic DNA. A, BamHI restriction map according to the work of Koike et al. (1). The 3′-part of the gene is not drawn to scale. The distribution of hPVR exons is given schematically in roman numerals. B, fine mapping of the upstream half of the 5.7-kbp BamHI fragment harboring the hPVR promoter. The BamHI-SacI fragment was sequenced and the SmaI site (2.88 kbp) and the SacI (2.9 kbp) map within the 5′-portion of cDNA clones H20A and H20B (2). Shaded boxes, Alu-repetitive elements. Open box, 5′-part of cDNA clone H20B. The XhoI site determining the 5′-end of the hPVR promoter sequence in Fig. 4 is given in bold letters.

Fig. 3. Northern blot with various cell line RNAs. 50 µg of each type of total cellular RNA were electrophoresed, and the amount of RNA in each lane was controlled by visualizing the ethidium bromide-stained RNA under UV light (not shown). The hybridizing probe was exon 2–6-containing cDNA fragment. For details see “Materials and Methods.”

most likely the reason for the apparent huge overproduction of hPVR RNA-specific signal. Indeed, on short exposure of the blot, only a single band was seen in JA-1 cells (Fig. 3), migrating to the same position as that from HeLa cells (data not shown). We conclude that hPVR mRNAs are approximately 3.5 kilobases in size.

The mRNAs for hPVRα, β, and γ share the same 3′-NTR whereas that of hPVRδ is different (Fig. 1B). Analyses with probes specific for the detection of the isoforms hPVRα, β, and γ or for hPVRδ revealed differences in the steady state levels of these two classes of mRNAs. Whereas hybridization with the former probe resulted in band intensities identical to those seen in Fig. 3, signals for the 3.5-kilobase-long hPVRδ mRNA were observed only on prolonged exposure of the Northern blots, regardless of the cell lines used (data not shown). However, the ratio of α, β, and γ versus δ messages is approximately the same in all cell lines tested, including JA-1.

Transcriptional Start Sites of the hPVR mRNAs—Initial experiments to map the start sites of hPVR mRNA by RNase protection or primer extension yielded inconsistent results in that putative start sites were spread over a relatively large area of the promoter (data not shown). Therefore, we used the 5′-RACE technique to determine the hPVR mRNA 5′-ends (26; see “Materials and Methods”). Sequence analysis of HeP2 RACE clones revealed the presence of multiple start sites for transcription within HeP2 cells (Table I and Fig. 4). Seventeen of 22 events mapped to either of three start sites within a 20-bp region (Fig. 4, closed circles). Minor start sites were suggested by a few clones mapping to the 3′-vicinity of the major start sites (Fig. 4, open circles). We then performed the same analysis using RNA isolated from the JA-1 cell line. It was of interest to us to examine the pattern of transcriptional start sites in JA-1 cells, in which the hPVR gene is transcribed in the mouse cell context, to determine whether it was similar to one we had mapped in HeP2 cells. As listed in Table 1, 10 of 25 RACE clones obtained from hPVR mRNA of JA-1 cells revealed start sites at the same locations as found in HeP2 cells. These data suggest that the 5′-ends of hPVR transcripts isolated from JA-1 cells were slightly more heterogeneous with respect to the start sites mapped in the HeP2 cell line (data not shown).

We had reported earlier the isolation of two cDNA clones (H20A and H20B) with 5′-NTRs longer than those determined by the 5′-RACE method (see Fig. 4 for the location H20B). Therefore, we used RT-PCR to determine whether transcriptional initiation events took place in the region upstream of the major start sites detected by 5′-RACE (see “Materials and Methods”). Primer C (Fig. 4, arrow) yielded a product with mRNAs of HeP2, HeLa, COS-1, and JA-1 cells, whereas primer B did not (data not shown). The start sites detected by RT-PCR upstream of the major initiation sites seemed to be used at a low frequency, because the uncloned HeP2 and JA-1 RACE pools could not be cleaved to any noticeable extent with restriction enzyme EaeI (see Fig. 4).

Inspection of the DNA sequence upstream of the start site cluster (Fig. 4) did not reveal TATA- or CAAT-like elements that are typical signals for precise initiation of transcription (27). Moreover, the sequences in the vicinity of the transcriptional start sites were found to have a much higher than average GC content. Many genes with these characteristics often harbor multiple start sites of transcription (28, 29). Therefore, our observation that the hPVR gene possesses multiple transcriptional initiation sites would be consistent with these previous investigations.

Location of the hPVR Promoter—To determine the sequences that are essential for promoter activity, an XhoI-ATG fragment (Fig. 5, fragment H) was generated by PCR and used for further analysis. Earlier results suggested that sequences upstream of the XhoI site are dispensable for basic promoter activity (CAT
reporter assays; data not shown). By PCR, a number of 5'- and 3'-deletions were introduced into the XhoI-ATG fragment, and the resulting DNAs (Fig. 5, 5'-deletions, A–D; 3'-deletions, E, F, G20, and G17) were cloned into a luciferase reporter vector. The results of transfections of the deletions constructs into HEp2, HeLa, COS-1, and L929 cells are shown in Fig. 6A. It is apparent that the activity pattern caused by the deletions is very similar for the four cell lines tested. This indicates that the hPVR promoter activity is controlled in a similar fashion in cells of human, monkey, and mouse origin. A significant decrease of activity was found as result of deletions from B to C or from E to F. Thus, a minimal DNA fragment possessing core promoter activity under the conditions of the experiment is the B to E sequence. Accordingly, the BE segment (Fig. 5, from –343 to –58) was cloned into the luciferase expression vector and was shown to possess full activity in the different cell lines (Fig. 6A). The 5'-end of the BE segment is located some 150 bp upstream of the major transcriptional start site cluster, whereas the 3'-end maps to 56 bp upstream of the translation initiation AUG codon. Taken together, these data suggest that sequences important for basic promoter activity reside in the region of the transcriptional start site cluster as well as within the region encoding the 5'-NTR of hPVR mRNA.

It is known that certain cell lines of the hematopoietic lineage cannot be infected with poliovirus (30, 31). Most likely, these cells do not express hPVR polypeptides. As indicated before, we have tested 13 BL cell lines, representing all three classes of translocation types (32) for the presence of hPVR RNA by Northern blotting. None of these cells showed detectable levels of a signal indicative of hPVR mRNA. Raji cells, one of the BL cell lines tested by Northern blotting, was further analyzed for hPVR expression by fluorescence-activated cell sorting analysis. Using hPVR-specific monoclonal antibodies, we were unable to detect hPVR at the surface of these cells, as expected (data not shown). On the other hand, Southern blot analysis has shown that the hPVR gene is not deleted in Raji cells (data not shown). Therefore, the lack of hPVR expression in Raji cells may be due to an inactive hPVR promoter. To test this hypothesis, we have transfected the H and BE promoter constructs into Raji cells. As can be seen in Fig. 6B, Raji cells were unable to support transcription from the exogenously transfected H (and BE, data not shown) promoter construct, a result in agreement with the lack of endogenous hPVR mRNA in these cells.

As seen in Fig. 6B, expression of luciferase under the control of the H fragment promoter is relatively low in COS-1 cells. This may be explained by the fact that the expression of luciferase from the positive control vector is driven by the SV40 promoter and enhancer, which is particularly strong in these cells.

Cloning of the Monkey PVR Promoters—In contrast to humans, monkeys possess at least two genes encoding PVRs.
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Fig. 5. Schematic drawing of the hPVR promoter region starting at XhoI. Numbering, A of ATG = +1. Open box, transcribed region with a biased line indicating the start site cluster. Thin lines indicate the sequences present in the 5'-deletion (A-D) and 3'-deletion (E-G17) constructs. The exact location of the borders of each fragment is indicated in Fig. 4.

<AGMa1 and AGMa2> (7). On the basis of the extent of the sequence divergence between the two genes, it is likely that a duplication of the monkey PVR gene occurred after species differentiation (7). Sequence homologies suggest that the hPVR proteins are more closely related to the corresponding protein encoded by AGMa1 (90.2% identity) than to those encoded by AGMa2 (86.4% identity) (7). The nonpathological function of any of these proteins is yet to be determined, but it is possible that mPVRa1 and mPVRa2 may have different functions or that the expression of these proteins may be regulated via their promoters in a different manner. These considerations prompted us to isolate the monkey promoters.

Oligodeoxynucleotides located in the 5'-coding region of mPVRa1 and mPVRa2 mRNAs, derived from the published sequence (7), and a 5'-oligonucleotide spanning the XhoI site (see Fig. 2B) were used to PCR amplify sequences corresponding to the human H fragment of COS-1 genomic DNA. As a control, a similar experiment was performed with HEp2 genomic DNA, yielding the H fragment (Fig. 5). The HEp2 PCR product was sequenced, and no differences were found when compared with the sequence of the original λ-human DNA insert. The promoters of both the AGMa1 and AGMa2 genes were sequenced (Fig. 4, alignment to hPVR). AGMa1 clones showed heterogeneity at three positions (designated Y, Y, and W), a finding that was confirmed by a second, independent round of PCR amplification and sequencing. A triplet, CTT, at position −552 of AGMa1 was also found in one of six clones of AGMa2. It is apparent that the degree of conservation between the promoter sequences is approximately the same as found for the coding regions of the corresponding mRNAs.

The AGMa1 and AGMa2 fragments generated by PCR were cloned into the luciferase reporter vector and found to exert promoter activity to an extent similar to the human counterpart (data not shown). Since RT-PCR of COS-1 mRNA, using the same primers as for human mRNAs (see above), suggested a transcriptional start site region in COS-1 cells similar to that found in human cell lines, we assume that the transcriptional control exerted by the monkey promoters is similar to that of their human counterpart. This conclusion, however, must await confirmation by 5'- and 3'-deletion experiments of the two monkey promoters.

It should be noted that the region upstream of the 5'-NTRs is particularly well conserved among all three promoters (Fig. 4), whereas the region preceding the major start sites harbors a hot spot of sequence divergence between the monkey and human promoters. In light of the finding that the upstream sequences are largely dispensable for basic hPVR promoter function, this was unexpected. However, the potential importance of these sequences for fine tuning of promoter activity was already suggested by the 5'-deletion analyses; the A and B deletions caused an increase of activity when transfected into primate cells and a slight but constant decrease on transfection into mouse L cells (see Fig. 6).
DISCUSSION

Typical infections of poliovirus in humans, initiated by ingestion of the virus, are restricted to cells of gastrointestinal organs. Only rarely (1% of all infections) does the virus reach the central nervous system, where it destroys motor neurons with great preferences, thereby causing a disease syndrome called poliomyelitis. Despite this apparent tropism, mRNA encoding hPVR can be detected in small quantities in many human tissues (reviewed in Refs. 21 and 22). The control mechanism(s) governing poliovirus tissue tropism is not understood. It may be related to patterns of expression of hPVR both at transcriptional (22) and translational (33) levels, as it may also involve cellular factors such as CD44 (34).

The current study has been undertaken to dissect the promoter region of the hPVR gene. Understanding the regulation of expression of hPVR proteins may not only be crucial to understanding the unique poliovirus pathogenicity, it may also shed light on the nonpathogenic function of these proteins belonging to a new human gene family.

All subtypes of mRNAs encoding mPVRs are found in low quantities in human tissue culture cells. Additionally, hPVR mRNA is much less abundant than hPVR-type mRNAs in all cases tested (data not shown). Whether the low steady state level of the hPVR message is due to less frequent splicing events or is the result of decreased hPVR mRNA stability (2) remains to be determined. Unexpectedly, this is not reflected by the amount of the corresponding proteins observed in various cell lines (10). The reason for the unequal expression of the hPVR mRNAs, on the one hand, and the roughly similar abundance of the polypeptides, on the other hand, is not known.

Using the RACE procedure, we have found that transcription of hPVR mRNAs initiates at multiple sites with a prevalence at two or three sites (Fig. 4, closed circles). This result is likely to be correct because: 1) two independent experiments using two different cell lines (HEp2 and JA-1) revealed similar results; 2) all three start sites match to some degree the consensus sequence for eukaryotic transcriptional initiation sites (CAG/TT) (27, 35); and 3) a modified RACE procedure (26) was also used to exclude that the results were derived from degraded RNA or products of incomplete reverse transcription. In this modified procedure, a RNA oligonucleotide is ligated to decapped mRNA molecules before reverse transcription is performed such that only full-length cDNA molecules can be amplified by subsequent PCR amplification. Moreover, degraded RNA is subjected to a dephosphorylation reaction before decapping of mRNAs is performed.

Minor start sites were observed by RT-PCR experiments, suggesting that a minority of transcripts originate from the region extending approximately to position ~280 (Fig. 4). A start site window similar to that of hPVR transcripts was also found for transcripts of mPVRa1 and mPVRa2 mRNAs by using the same 5′- and 3′-primers as for hPVR in the RT-PCR (data not shown).

Since the hPVR gene maps to chromosome 19, we have used a corresponding λ-phage library to search for sequences upstream of the first hPVR exon. A fragment of the hPVR gene, approximately 2.9 kilobases in length, was identified as containing promoter activity (Fig. 2B). Sequence analysis of this fragment and successive 5′-deletions suggested that the promoter activity resides downstream of the XhoI site (data not shown). Accordingly, the remaining sequence of the hPVR gene (XhoI to initiating AUG) was subjected to an intensive analysis revealing a minimal promoter fragment of approximately 280 bp that contained the transcriptional start site cluster.

The characteristics of the hPVR promoter (short minimal DNA sequence, relatively CG-rich, and lack of TATA- or CAAT-like sequences) are common to a variety of genes, such as the human phenylalanine hydroxylase (38), murine complement component C4 (37), mouse thymidylate synthetase (38), mouse mb-1 (28), mouse fibroblast growth factor receptor 1 (39), and human pregnancy-specific protein (16) genes and the human RET proto-oncogene (40). It is noteworthy that most of these genes show cell type and/or developmentally regulated expression.

Several cell lines derived from hematopoietic tissues, BL lines included, have been reported to be resistant to poliovirus infection (30, 31). Northern blot analyses for hPVR mRNA in RNAs of 12 BL cell lines were negative (data not shown). RNAs of BL40 and Raji cells did not yield a signal even by the RT-PCR method. On the other hand, transfection of poliovirus RNA into Raji cells leads to poliovirus replication, a result eliminating the possibility of a cell internal block to viral replication. Therefore, we conclude that resistance of these cells to poliovirus replication is determined by the failure to express a suitable receptor. Since an exogeneously transfected hPVR promoter is inactive in Raji cells (see Fig. 6B), the apparent lack of hPVR expression in these cells is likely to be due to transcriptional repression.

Preliminary footprint analyses, using HeLa cell extracts, revealed three areas that suggested protection (for location see Fig. 4; data not shown). A very similar footprint pattern was obtained when the experiment was carried out with nuclear extracts of mouse L cells (data not shown), a result suggesting that factors controlling hPVR promoter activity and transcriptional start site selection are similar in human and mouse cells. This was not unexpected, since mice transgenic for the hPVR gene are not only sensitive to poliovirus infection, but when inoculated intravenously or intracerebrally, they produce a disease syndrome nearly identical to human poliomyelitis (24, 25, 41, 42).

As determined by computer analysis (43), possible candidates for factors binding to the hPVR core promoter region are PuF, AP2, and GCF factors. AP2 and GCF binding sites have been observed in promoters related to the structure of the hPVR promoter, such as the human ATP synthase α subunit (44), rat SSTR1 (45), rat SSTR4 (46), human ret proto-oncogene promoters (40), and mouse fibroblast growth factor receptor 1 (39). Gel mobility shift analyses needed to further characterize the binding activities to the hPVR promoter are in progress. It will be particularly interesting to test whether the expression of the hPVR gene is developmentally regulated or modulated in specific differentiation pathways, since, besides the AP2 sites, two hypothetical PEA3 sites are apparent in the hPVR promoter sequence. PEA3 motifs have been observed in promoters of developmentally regulated genes (for example, see Ref. 47).

We have also isolated and analyzed the promoter regions of the AGM genes, the monkey homologues to hPVR. These promoters are highly homologous to that of the hPVR gene with the exception of the region upstream of footprint 3. The significance of this difference is not known.

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