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Restricted variability of a 17 nucleotide stretch within the 5′-noncoding region of poliovirus genome

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SUMMARY

The outbreak of poliomyelitis in Finland in 1984 was caused by a wild strain of poliovirus 3 with uncommon molecular and antigenic properties. We prepared a synthetic oligonucleotide probe complementary to nucleotides 494–510 in the 5′-noncoding part of the genome of a representative strain of the outbreak. This short nucleotide stretch was found to be relatively well conserved within the outbreak and uncommon among 82 independent poliovirus isolates. It may thus be a useful marker for screening isolates to identify those requiring more detailed genetic comparison. The sequences of the corresponding region of the genome are known for 32 separate poliovirus strains and 3 coxsackie B virus strains and show 6 fully conserved nucleotides that could assume a constant hairpin-loop position in a hypothetical secondary structure of the RNA. This could explain the persistence of a particular 17 nucleotide sequence for 40 years in nature in this highly variable region of the poliovirus genome.

INTRODUCTION

An outbreak of poliomyelitis due to a wild type 3 strain of poliovirus occurred in Finland in 1984–5 (1). This was unexpected because the country had been free from the disease for 20 years and no signs of poliovirus circulation in the population had been obtained in thorough studies (2). The antigenic properties of the virus strains isolated during the outbreak have been shown to differ considerably from those of the type 3 strains (Sabin 3 and Saukett) that are used in poliovirus vaccines (3–5). Sequencing of the total genomic RNA of one of the isolates revealed several interesting differences from the 'reference' type 3 strain (P3/Leon) (6). These included a region in the 5′-noncoding part of the genome with a nucleotide sequence identical to that of type 1/Mahoney strain of poliovirus and spanning 16 (7) or 17 (8) nucleotides. Only 8 of the 17 nucleotides show identical positions with those of the type 3 Leon strain (9).

We wanted to study whether this unexpected intertypic relationship is shared by the other strains isolated during the outbreak and whether this property could be used as a marker in the search for the origin of the outbreak. In this paper we describe initial studies based on screening poliovirus isolates for RNA sequence

relationships and present a hypothesis for the persistence of a particular sequence in this highly variable region of the poliovirus genome.

MATERIALS AND METHODS

Virus strains

Eighty-eight out of 161 virus strains used in this study were isolated from the patients with poliomyelitis or healthy contacts of the patients, or their contacts, during the outbreak in Finland in 1984. Additional wild type strains of poliovirus 1, 2 and 3 originally obtained from different parts of the world are listed in Table 1.

All strains were serotyped by neutralization with type-specific hyperimmune animal sera using either a stock virus preparation or after plaque purification (see Results). Some strains were examined for Sabin strain-specific antigenic properties using microneutralization as described by Huovilainen and colleagues (3).

Strains designated P3/Saukett were kindly provided by Dr P. D. Minor, National Institute for Biological Standards and Control, London. The other laboratory strains tested were originally obtained from the Regional Enterovirus Reference Laboratory, State Serum Institute, Copenhagen, and had been maintained for varying periods with various passage histories in the Enterovirus Laboratory, National Public Health Institute, Helsinki.

Preparation of specimens for hybridization

Monolayer cultures of Vero cells were infected at low multiplicity. The infected cell cultures were harvested when the cytopathic effect involved 80–100% of the cells. The cellular membranes were lysed with the nonionic detergent Nonidet P-

Table 1. *Poliovirus strains used in the study*

Strain	Year	Country of origin
P1/125/1/1	1971	Czechoslovakia
P1/157/1/10	1971	USA
P1/178/2/7	1972	Poland
P1/Atlanta 1–1	1957	USA Arkansas
P1/Atlanta 77675	1955	USA Hawaii
P1/Atlanta 04958	1977	Kuwait
P1/Atlanta 13021	NK	Brazil
P1/Atlanta 5/78	1978	Morocco
P1/Atlanta 7/79	1979	Morocco
P1/98/82	1982	Italy
P1/12127	1976	Greece
P1/NE-459/82	1982	Spain
P1/Braz/8–3827/81	1981	Brazil
P1/Rom/53903/81	1981	Romania
P1/HK/15/81	1981	Hong Kong
P1/11FJ	NK	Sweden/India
P1/1400	1980	Mexico
P1/4969	1983	Saudi Arabia
P1/18389	1976	UK
P1/2069/77	1977	UK
P1/2	NK	Finland

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Table 1. (*cont.*)

Strain	Year	Country of origin
P1/3–3855/62	1962	Finland
P1/4–X1/56	1956	Finland
P1/5–4032/62	1962	Finland
P1/7–TF/60	1960	Finland
P1/9–TJ/59	1959	Finland
P1/11/59	1959	Finland
P1/12–1113 L/60	1960	Finland
P1/13–3848/62	1962	Finland
P1/14/59	1959	Finland
P2/172/2/7	1972	Poland
P2/185/2/3	1973	UK
P2/II–215	1959	Venezuela
P2/II–299	1952	USA California
P2/II–316	1952	Egypt
P2/II–364	1956	India Bombay
P2/Lennette 77726	1954	USA California
P2/76/78	1978	Morocco
P2/92/78	1978	Morocco
P2/4141/82	1982	UK
P2/II–867	1974	USA Maryland
P2/273/82	1982	Spain
P2/81–4789	1981	Korea
P2/B 1139	1982	Kuwait
P2/LS 2575	1980	Kuwait
P2/5394	1983	Saudi Arabia
P2/Eng/23/84	1984	England
P2/282/N/84	1984	Spain
P2/121/84	1984	Spain
P2/967/N/84	1984	Spain
P3/177/2/7	1972	Poland
P3/III–1	1957	USA New Mexico
P3/III–2	1958	USA Indiana
P3/III–5	1958	USA Washington DC
P3/III–10	1957	USA Georgia
P3/III–374	1957	Japan
P3/III–715	1958	India Bombay
P3/Lennette 77750	1953	USA California
P3/137/1976	1976	Hungary
P3/109/76	1976	Morocco
P3/40/82	1982	Spain
P3/Ext 2672	1980	France
P3/82–18714	1982	Turkey
P3/1838/83	1983	Spain
P3/1293/83	1983	Spain
P3/1573/83	1983	Spain
P3/E684	1957	Sweden
P3/476/62	1962	UK
P3/6/62	1962	UK

NK = not known.

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40 as 0·5% in phosphate buffered saline and the lysates were kept on ice for 10 min. They were then gently vortexed and the nuclei were pelleted at 1000 g for 10 min. The supernatant was removed and treated with proteinase K (Merck AG, Darmstadt, Federal Republic of Germany) at a concentration of 0·1 mg/ml for 2 h at 37 °C. The RNA in the specimens was usually probed without further purification. For certain purposes (see Results) the proteinase K-treated preparations were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and used as such or after precipitation of the RNA with ethanol. The RNA in the specimens was denatured by adding formaldehyde to a final concentration of 6·5% (v/v) and incubating for 15 min at 65 °C. Then 20 × SSC (1 × SSC = 0·15 M NaCl, 0·015 M sodium citrate, pH 7·0) was added to make a final concentration of 6 × SSC. Samples representing 10^6 cells were spotted onto nitrocellulose filters (BA 85, Schleicher & Schuell Co., Dassel, Federal Republic of Germany) with a micro-sample filtration manifold (Minifold II, Schleicher & Schuell Co.). After air-drying the filters were baked at 80 °C for 2 h.

Labelling of oligonucleotide

The synthetic oligonucleotide (5′-AATCACTGGTTTGTGAC-3′) was purchased from the Department of Medical Chemistry, University of Helsinki and labelled according to the manufacturers instructions at the 5′ end with ^{32}P using bacteriophage T4 polynucleotide kinase (Boehringer, Mannheim, Federal Republic of Germany). The reaction mixture (50 μl) contained 10 pmol of the probe, 0·1 M Tris-HCl (pH 7·6), 20 mM $MgCl_2$, 0·2 mM EDTA, 50 mM dithiotreitol, 10 U of kinase, 50–75 μCi (γ-^{32}P)ATP (> 5000 Ci/mmol, Amersham International Plc, England). Sterile distilled water was added as necessary to make the final volume 50 μl. After 1 h incubation at 37 °C the kinase was denatured by heating for 10 min at 65 °C.

Labelled oligonucleotide was separated from free (γ-^{32}P)ATP by chromatography on Sephadex G-25 and used directly in hybridization. The specific activities of the probe were in the range $3–9 \times 10^8$ cpm/μg.

Hybridization assay

The filters were hybridized in a plastic bag with the ^{32}P-labelled probe (10^6 cpm/ml) in 5 × SSC, 5 × Denhardt's solution, 0·1% sodium dodecyl sulphate (SDS) for 4 to 16 hr at $T_m - 8$ °C. T_m in °C was estimated by the formula $T_m = (A+T)2 + (G+C)4$. After hybridization the filters were washed three times for 5 min in 5 × SSC, 0·5% SDS at T_m. Washed filters were exposed to Kodak X-Omat AR film with two intensifier screens for 16–48 h.

Virus purification for RNA sequencing

Viruses were grown in HEp-2 cells in the presence of ^{35}S-methionine and the cells were collected when a cytopathic effect was observed in 80–100%. The cells were lysed with nonionic detergent Nonidet P–40 (0·5% in PBS) and kept on ice for 10 min. The cell homogenate was slightly vortexed and cleared of cell debris by centrifugation for 10 min at 1000 g at 4 °C. Sodium dodecyl sulphate (SDS) was added to the supernatant to a final concentration of 0·5%. The virus was pelleted from the supernatant by layering it over a 2 ml 20% sucrose cushion and

centrifuging at 38000 rpm for 2 h at 15 °C in an SW40 rotor (Beckman Instruments, Inc.). The virus was further purified by velocity sedimentation in a 15%–30% sucrose gradient at 38000 rpm for 1·5 h in an SW40 rotor and pelleted from the peak radioactive fractions.

Extraction of virion RNA

Purified virus was diluted in 0·15 M sodium acetate and 0·1% SDS. The mixture was extracted sequentially with phenol-chloroform-isoamyl alcohol (25:24:1), phenol, chloroform and ether. The RNA was then ethanol-precipitated, dried and dissolved in water at a concentration of approximately 1 μg/μl. 5 U of RNase inhibitor (Boehringer, Mannheim) per μg was added.

Primer extension sequencing

The viral RNA was sequenced directly by the dideoxynucleotide chain termination method of Sanger and his coworkers (10). The primer used had the sequence 5′ GTAGTCGGTTCCGCC 3′ and was synthesized at the Department of Medical Chemistry, University of Helsinki. The hybridization mixture contained approximately 1 μg of template and 20 ng of primer. The mixture was heated at 90 °C for 1 min and allowed to cool to room temperature. Then 2·5 μl of water and 0·5 μl of reverse transcription buffer (final concentration: 50 mM Tris-HCl pH 8·3, 6 mM $MgCl_2$, 40 mM KCl) were added. One μl of this mixture was added to each of four tubes containing 1 μl of deoxynucleotide/dideoxynucleotide mixture, 8 μCi of ^{35}S-labelled dATP(> 1000 Ci/mmol, Amersham Corp., London, England) and 5 U of reverse transcriptase (P & S Biochemicals Limited, Liverpool, England) in a final volume of 3 μl. The A reaction contained 55·5 μM each dCTP, dGTP, dTTP and 1·4 μM dideoxy ATP. The C reaction contained 55·5 μM each dGTP and dTTP, 11·1 μM dCTP and 2·8 μM dideoxy CTP. The G reaction contained 55·5 μM each dCTP and dTTP, 11·1 μM dGTP and 2·8 μM dideoxy GTP. The T reaction contained 55·5 μM each dCTP and dGTP, 11·1 μM dTTP and 2·8 μM dideoxy TTP. Each reaction mixture was incubated at 42 °C for 30 min and then a chase mix containing 250 μM each dATP, dCTP, dGTP and dTTP in 100 mM Tris-HCl pH 8·3, was added and incubation continued for 15 min. The reaction was stopped by adding 5 μl of formamide-dye mix. The specimens were heated for 2 min at 90 °C before loading onto 6% polyacrylamide buffer gradient gel (11). After electrophoresis the gel was soaked for 10 min in 10% acetic acid, 10% methanol and dried at 80 °C under vacuum before autoradiography (Hyper film β max, Amersham Corp., England) for 12–48 h.

RESULTS

Sensitivity and specificity of oligonucleotide hybridization

To evaluate the sensitivity and specificity of the test, serial dilutions of the RNA prepared from P1/Mahoney, P3/Leon or P3/Sabin infected or uninfected Vero cells were probed with the labelled oligonucleotide complementary to the sequence on P1/Mahoney strain. The probe regularly detected RNA from 10^4 homologously infected cells and showed no cross-reactions with preparations derived from the Leon or Sabin 3 infected or uninfected Vero cells (Fig. 1).

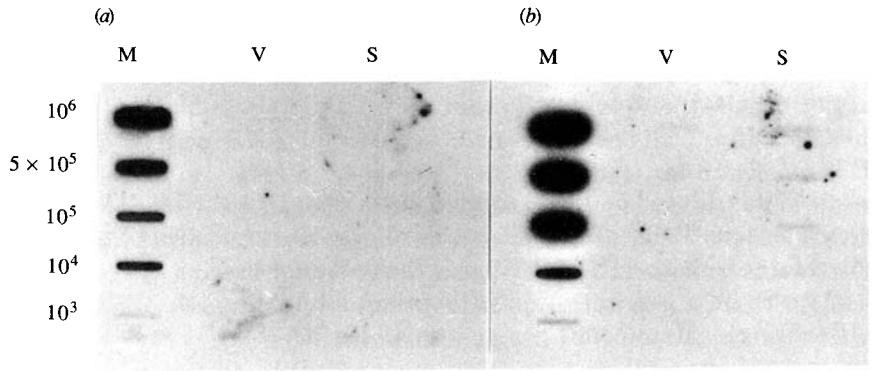

Fig. 1. Sensitivity of the oligonucleotide hybridization reaction. Proteinase K- treated (*a*) cytoplasmic extracts of Vero cells infected with P1/Mahoney (M), P3/Sabin (S) or uninfected Vero cells (V) were spotted on nitrocellulose filters and tested with the P1/Mahoney probe. The corresponding cell numbers are shown on the left. In panel *b* the proteinase K-treated extracts were deproteinized with the phenol-mixture and probed as in panel *a*.

Homology within an outbreak

To test the stability of the RNA sequence in this region within an outbreak, poliovirus type 3/Finland/84 strains isolated from 88 persons were grown in Vero cells and the proteinase K-treated cytoplasmic extracts were probed with the oligonucleotide. All virus strains were recognized by the Mahoney probe. Six strains showed relatively weak, fuzzy and variable signals in the screening test (for instance C15 in Fig. 2). Ethanol-precipitated RNA preparations were, however, undoubtedly positive for the Mahoney probe (data not shown).

Variation among independent poliovirus isolates

To test the variation of this region among poliovirus strains in general we first assayed 12 laboratory strains. Apart from the expected reactions only one strain, P1/Charleston, could be detected with the Mahoney probe. It is noteworthy that P2/Sabin, which differs from the Mahoney strain by only three nucleotides (Table 4), showed no signal with the Mahoney probe (Table 2).

Ten wild type 1 strains isolated in Finland in the 1950s were negative with the probe. Sixty strains, 20 of each serotype, from the collections of National Institute for Biological Standards and Control, London, UK, isolated in various regions in the world between 1952 and 1984 were also tested. Six strains were positive with the Mahoney probe and these included representatives from all serotypes (Table 3). Three out of four probe positive type 1 poliovirus isolates showed Sabin-like antigenic properties in the microneutralization test.

The latter 60 strains were tested both before and after phenol extraction. As with the P3/Finland/84 strains, the specimens showing a weak, fuzzy signal when tested as a crude preparation, were clearly positive when tested after phenol extraction (Fig. 3). Use of phenol-extracted RNA did not increase the number of strains scored as positive.

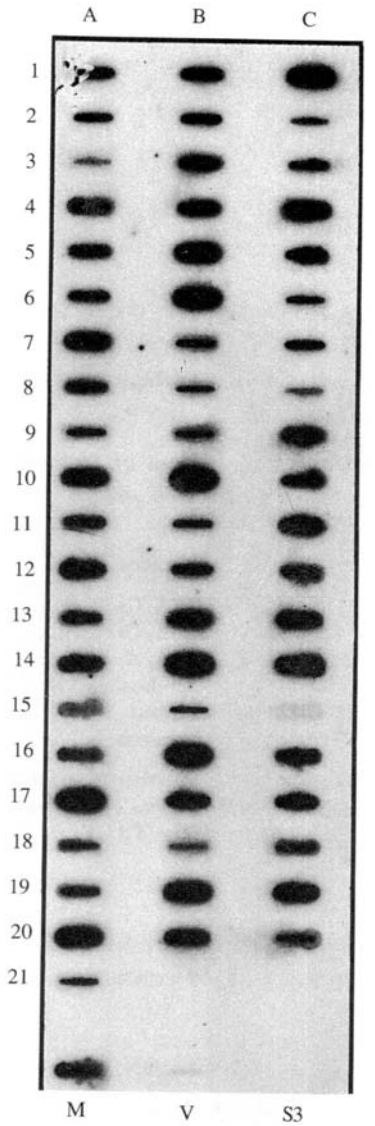

Fig. 2. Homogeneity of type 3 poliovirus strains isolated in Finland in 1984. Proteinase K-treated cytoplasmic extracts of about 10^6 Vero cells infected with 61 different strains of type 3 poliovirus isolated in Finland in 1984 were spotted on nitrocellulose filters and tested with the Mahoney probe (columns A, B and C). M, P1/Mahoney; V, uninfected Vero cells; S3, P3/Sabin.

Variation of nucleotide sequence in the probed region of RNA

Genomic RNAs from five strains showing a positive signal with the Mahoney probe and 10 non-reacting strains were sequenced using the primer extension method. Results in Table 4 indicate that, among this limited member of isolates, mismatches greater than one nucleotide were not accepted by the hybridization test. Another aspect evident from Table 4 is that the 17 nucleotide stretch probed

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Table 2. *Hybridization results of 'laboratory strains' with the Mahoney probe*

Virus strain	Results
P1/Brunhilde	−
P1/Charleston	+
P1/Mahoney	+
P1/Sabin	+
P2/MEF-1	−
P2/Sabin	−
P3/Leon	−
P3/Sabin	−
P3/Saukett*	−

* Seven strains maintained at different laboratories were tested separately.

Table 3. *Positive hybridization reactions out of 70 unrelated poliovirus isolates tested (Mahoney probe)*

Virus strain	Year	Country	Location in Fig. 3
P2/II–299	1952	USA/California	B4.E4
P3/Lennette 77750	1953	USA/California	C8.F8
P1/Atlanta 1–1	1957	USA/Arkansas	A4.D4
P1/125/1/1*†	1971	Czechoslovakia	A1.D1
P1/178/2/7†	1972	Poland	A3.D3
P1/76/78†‡	1978	Morocco	B8.D22

* Strains were isolated from patients who had recently received OPV.
† These strains showed Sabin 1-like immunological characteristics.
‡ This is a plaque purification derivative of the P2/76/78 strain that turned out to be a mixture of type 1 and type 2 polioviruses.

in the above experiments is indeed highly variable between independent poliovirus isolates. However, it contains a set of six conserved nucleotides C-A-CCAG.

DISCUSSION

We have used an oligonucleotide hybridization test for initial screening of RNA sequence relationships between poliovirus isolates. Our results reported in this paper show that a 17 nucleotide sequence in the 5′-noncoding region that is identical in type 1/Mahoney and type 3/Finland/23127/84 strains, is also possessed by all 88 other type 3/Finland/84 strains tested but is relatively rare among other unrelated type 3 poliovirus isolates. The probe thus seems to be suitable for searching for possible genetic relatives of the P3/Finland strain. The method is relatively easy to use for large numbers of specimens since proteinase K-treated cytoplasmic extracts gave practically identical results with phenol-extracted specimens.

Oligonucleotide hybridization is a sensitive test capable of detecting one nucleotide mismatch provided that the stringency of the assay conditions is high enough (13, 14). Since we wanted to develop an assay for detecting relatedness between virus isolates, a mismatch of one or two nucleotides would still be

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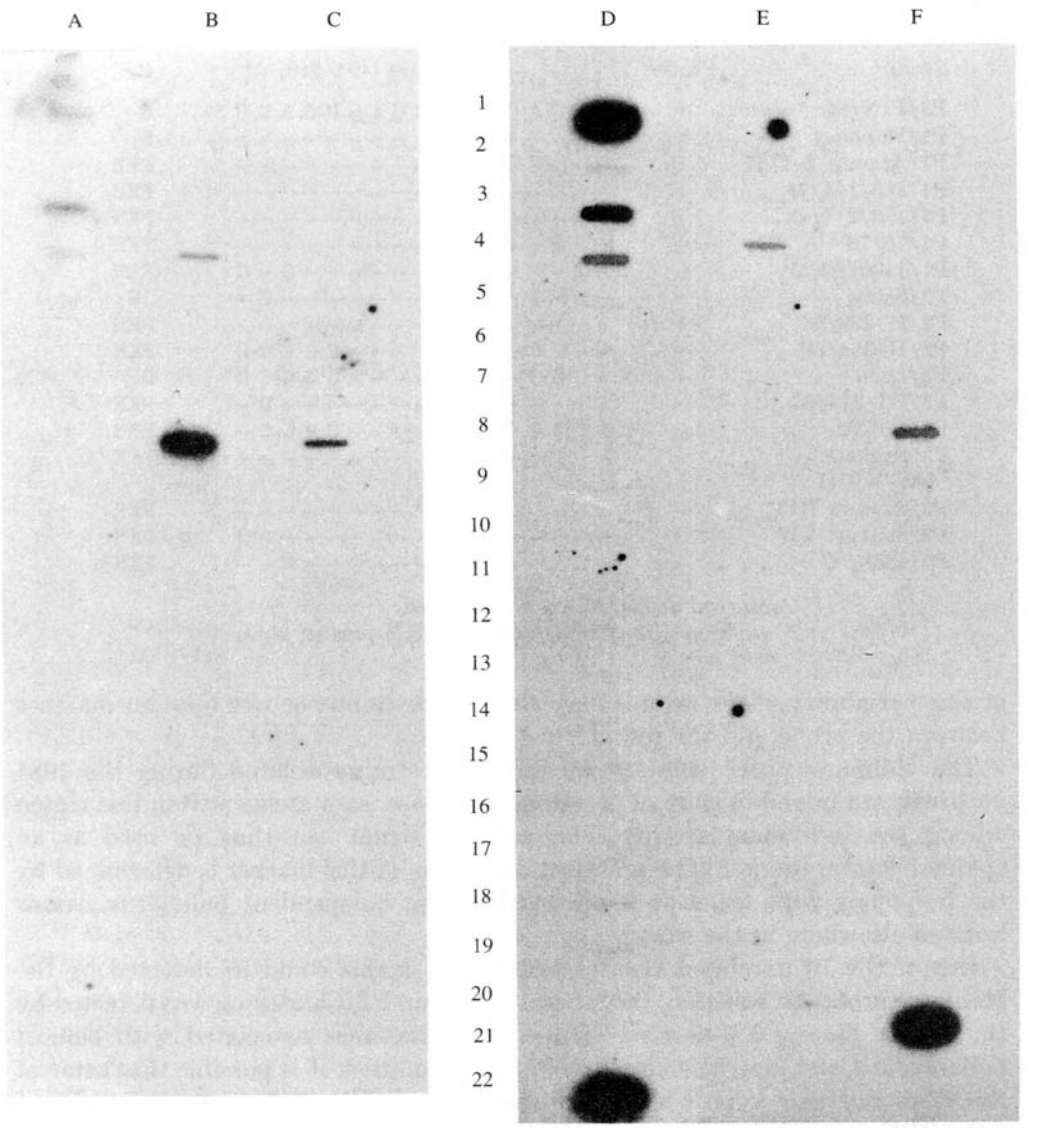

Fig. 3. Distribution of P1/Mahoney-related sequences among 60 unrelated poliovirus isolates (see Table 1). For details see legend for Fig. 2. Columns A to F, reactions with the Mahoney probe. Columns D to F show confirmatory experiments with phenol-extracted specimens and the Mahoney probe. The specimens are as in A to C except that B8 was translocated to D22 and P1/Mahoney control was added to location F21.

acceptable. Sequencing the genomic RNA of selected strains in this region suggested that this indeed was the case. Of course, the location of the mismatch may influence the results but a greater number of sequences would be required to evaluate this. The intensity of the positive signal varied from strain to strain, although we attempted to use a standard amount of infected cells per specimen. The amount of RNA synthesized per cell may vary between virus strains but part

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Table 4. *Known nucleotide sequences in the probed region of poliovirus RNA*

Strain	Probe*	Sequence (494–510)	Ref.
P3/FIN/84	+	5′ G U C A C A A A C C A G U G A U U 3′	6
P1/Mahoney	+	– – – – – – – – – – – – – – – – –	8
P1/Atlanta 1–1/57	+	– – – – – G – – – – – – – – – –	PKH
P1/125/1/1/71	+	– – – – – – – G – – – – – – – –	PKH
P1/178/2/7/72	+	– – – – – – – – – – – – – – – –	PKH
P1/76/78	+	– – – – – – – – – – – – – – – –	PKH
P1/1400/80	–	A C – – – – – G – – – – – – G – C	PKH
P2/Sabin	–	– – – G – G – – – – – – – – – C –	12
P2/II–299/52	+	– – – – – G – – – – – – – – – –	PKH
P2/II–364/56	–	– – U G – – – C – – – – C A – C C	PKH
P3/Leon	–	– C U G – – – C – – – – C A G C C	9
P3/III–374/57	–	A – – – – – – G – – – – – – – C –	PKH
P3/476/62	–	A C U G – – – – – – – – C A G C –	PKH
P3/Saukett BW/NPHI	–	A – – – – – – G – – – – – – – – –	PKH
P3/Saukett RIT	–	A – – – – – – G – – – – – – – – –	PKH
P3/Saukett VW	–	A – – – – – – U – – – – – – – – G	PKH
P3/1838/83	–	– C – – – – – – – – – – – – G – –	PKH

Conserved nucleotides are underlined.
*, reaction with Mahoney probe; PKH present study.

of this variability of the signals may also be due to one or two base mismatches between the probe and the tested viral genome.

The Mahoney probe detected all tested 88 strains isolated during the 1984 outbreak in Finland in spite of occasional one-base mismatches within this region among the individual strains. The positive signal can thus be used as an epidemiological marker. The practical usefulness of this marker is determined by the frequency with which it is observed among independent poliovirus strains isolated elsewhere in the world.

Out of the 70 unrelated isolates tested, six strains could be detected by the Mahoney probe. In addition, the laboratory strain P1/Charleston was detected by the probe. Since OPV-derived viruses are sometimes associated with clinical poliomyelitis and may also circulate in the population, it is possible that some of the 'Mahoney-like' type 1 isolates in this kind of strain collection may turn out to be OPV-derived descendants of Mahoney-Sabin 1. Indeed, three out of the four laboratory type 1 strains used in this study had Sabin 1-like antigenic properties. However, since the six Mahoney-probe positive strains also included representatives of all three serotypes isolated in USA in 1950s, we can conclude that this sequence, or a sequence very similar to it, is not confined completely to P1/Mahoney and P3/Finland/84 strains only. Whether the P3/Lennette/77750/53 strain which shares homology in this sequence with P1/Mahoney is an ancestor of the P3/Finland/84 strains remains to be shown by more detailed comparisons. It will also be interesting to see if strains sharing this sequence with P1/Mahoney and P3/Finland/84 can be found among wild type isolates from 1960s and 1970s.

While the results with the Mahoney probe were easy to score as either negative or positive we had technical difficulties with another probe complementary to the corresponding sequence of P3/Leon strain. The latter gave irregular results with

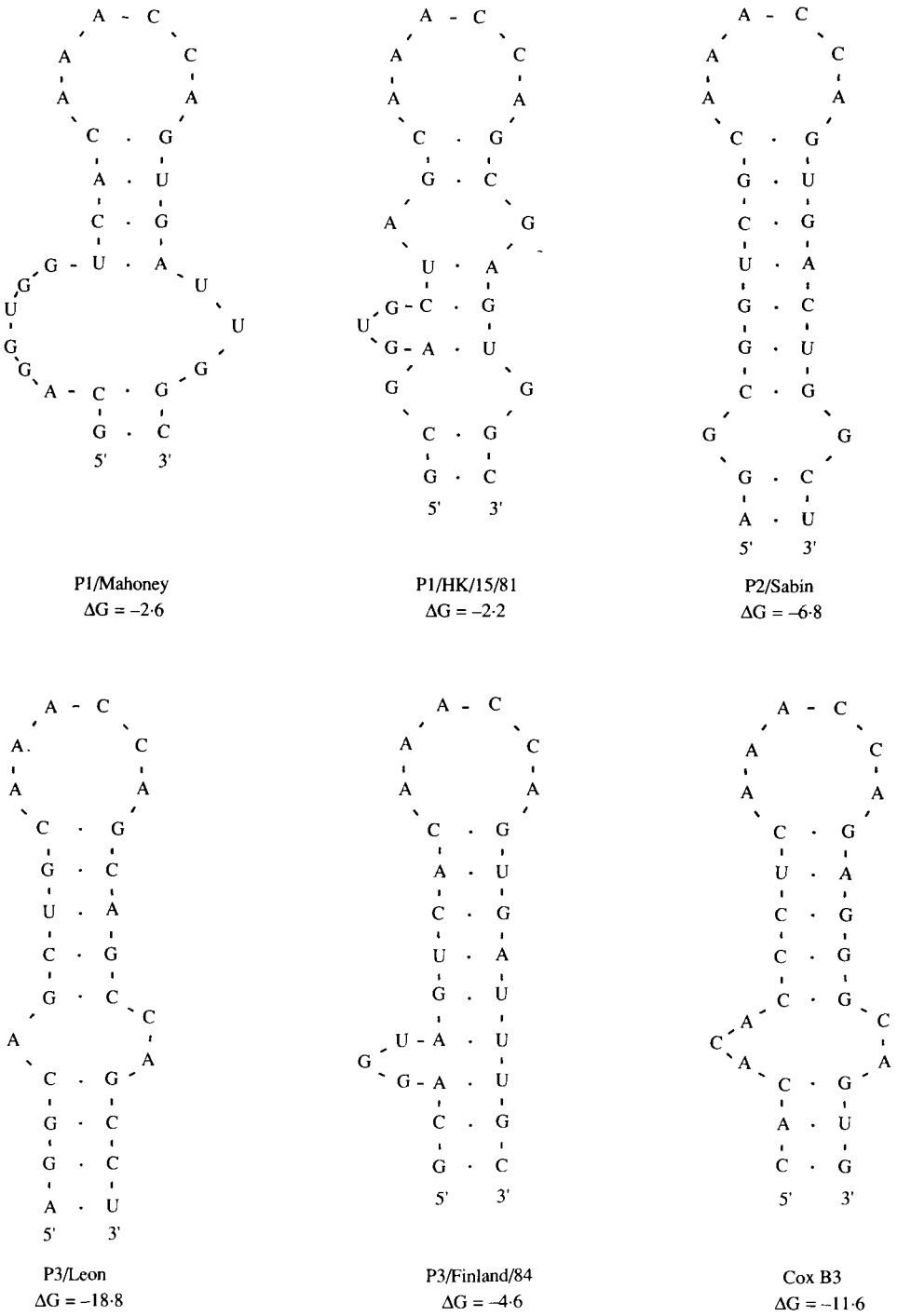

Fig. 4. Hypothetical base pairing close to the probed region in enterovirus genomes. The sequences are according to Racaniello and Baltimore (8, P1/Mahoney); Hughes et al. (6, P3/Finland/84); Toyoda et al. (12, P2/Sabin); Minor and Dunn (17, P1/HK/15/81); Stanway et al. (9, P3/Leon); Lindberg et al. (20, Cox B3). The calculated free energies for the suggested secondary structure are shown in the figure (23).

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both P3/Leon and P3/Sabin infected cells and reacted variably with uninfected cells (data not shown). Therefore, careful characterization of the reaction patterns of an oligonucleotide probe is necessary before it can be used in epidemiological studies.

There are two possible explanations for the existence of an identical sequence in the type 3/Finland/84 strains and the type 1/Mahoney strain isolated more than 40 years earlier. Firstly, a recombination between a wild type 3 strain and the type 1 Mahoney-derived attenuated Sabin 1 strain, which is widely used in oral polio vaccinations, cannot be ruled out. The other possibility is that certain sequence variations may be more favourable for the persistence of the strain than some others.

The exact RNA sequence of this region is known for 32 different strains of poliovirus (Table 4, Pöyry, Kinnunen and Hovi, unpublished; 15–17) and indicates wide variation without any serotype-dependence. Variability of the sequence does not necessarily mean lack of specific function since this region is known to be required for successful replication of poliovirus *in vitro* (18). Furthermore, a set of six nucleotides within this highly variable region was found to be fully conserved. Three serotypes of coxsackie B viruses also share this six nucleotide set (19, 20, 21). Computer-predicted secondary structure of the RNA of type 3 poliovirus of the Leon strain suggests that the region probed with our oligonucleotides is located close to the end loop of a 27 nucleotide hairpin structure involving nine base pairs (22). By taking into account possible base pairing close to this region alone it is possibly to construct an alternative model of the secondary structure which provides the conserved six nucleotides with a constant loop position in all 32 cases (Fig. 4). While this manuscript was in preparation, Rivera and coworkers published another computer model of the secondary structure of the 5′ noncoding region of genomic RNA suggesting a well-conserved pattern among various picornaviruses. The loop deduced from the sequences in the present study also exists in the computer model (24).

In conclusion, we have shown in this paper that a 17 nucleotide sequence shared by the P1/Mahoney and P3/Finland strains, is rare among independent type 3 poliovirus isolates while being relatively stable within the outbreak in Finland. A probe recognizing this region of RNA has therefore been used as one tool in further searchers for the origin of the outbreak. Since some strains of the two other serotypes were also recognized by this probe, other methods, such as partial RNA sequencing of selected regions of the genome, is being used in these studies as well.

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