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Molecular Typing of Enteroviruses: Current Status and Future Requirements

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INTRODUCTION

The enteroviruses comprise a large genus belonging to the *Picornaviridae*. Sixty-six immunologically distinct serotypes are known to cause infections in humans. Most infections are mild or asymptomatic, and as a result enteroviruses are considered by many to be unimportant as human pathogens and unworthy of sustained investigation in the light of other viral infections of greater perceived public health importance. However, enteroviruses may also result in serious or even fatal disease. Enteroviruses are the most commonly implicated viral agents of acute myocarditis and aseptic meningitis. Congenital infections occur, although their frequency is unknown. Infection in neonates is frequently life-threatening. The enterovirus group includes the polioviruses (PV), the cause of paralytic poliomyelitis, which still causes significant disability in many parts of the world. There is now growing evidence that enteroviruses also cause or contribute to common chronic diseases, including dilated cardiomyopathy, one of the commonest indications for cardiac transplantation, insulin-dependent diabetes mellitus, and chronic fatigue syndrome.

Typing of viruses is required primarily to provide information on the relationship between viruses, to identify virus types with increased virulence or specific disease attributes, for epidemiological investigations, and to allow correlation with viral immunity. New molecular technologies have spawned the development of molecular typing methods for several virus groups that are not amenable to other means of investigation and have raised the possibility of simpler typing methods for other virus groups that have traditionally been typed on a phenotypic basis.

Based on their ability to replicate in cells of human and primate origin, infectivity and pathogenicity in different animal species, and antigenic differences, human enteroviruses were originally subclassified into PV serotypes 1 to 3, coxsackievirus groups A and B (CAV, serotypes 1 to 22 and 24; CBV, serotypes 1 to 6) and echoviruses (ECV; serotypes 1 to 7, 9, 11 to 27, and 29 to 33) (reviewed in references 269 and 287). The value of these distinctions has to some extent been diminished by the availability of recently described cell lines permissive for a wider range of enteroviruses and by the intrinsic variability of biological phenotypes observed among naturally occurring enterovirus strains. Since 1970, newly identified serotypes have not been assigned to the above groups but, rather, have been numerically classified as enterovirus serotypes (ENV) 68 to 71 (279).

Enterovirus infection in humans may result in a wide range of acute symptoms involving the cardiac and skeletal muscle, central nervous system (CNS), pancreas, skin, and mucous membranes (Table 1) (reviewed in references 269 and 289). Poliomyelitis caused by PV has been successfully eliminated from many parts of the world by a successful World Health Organization (WHO)-sponsored Poliomyelitis Eradication Initiative (PEI), but other enterovirus infections remain frequent and sometimes serious causes of morbidity. Enterovirus infection has also been associated with such chronic diseases as dilated cardiomyopathy and chronic myocarditis (17, 49, 54, 197, 303, 305), chronic fatigue syndrome (16, 77, 136, 482), insulin-dependent diabetes mellitus (reviewed in references 25, 26, and 481), motor neuron disease (464), and postpoliomyelitis syndrome (400). Evidence from studies in murine models indicates that chronic enterovirus infection is characterized by restricted genome replication and gene expression, although some controversy about the role of enterovirus infection in chronic disease in humans remains (reviewed in reference 301). Chronic infections also occur in immunodeficient patients (264).

Due to the diversity of disease manifestations, few of which are unique to enterovirus infections, diagnoses cannot be made on clinical grounds alone. Although specific therapy for enterovirus infections is not yet available, diagnosis is nonetheless important to distinguish between enterovirus-induced disease and other treatable causes of clinically similar disease, to identify PV outbreaks, and to assess the prognosis. A number of potential benefits in patient management accrue from a specific diagnosis of enterovirus infection (reviewed in reference 67). Traditionally, enteroviruses have been detected by isolation in cell culture, and their serotypic identity has been established by neutralization of infectivity with serotype-specific antisera. However, recent developments in molecular detection technology make it probable that enterovirus diagnosis will increasingly be achieved by non-culture-based methods, particularly nucleic acid amplification methods such as PCR. This review assesses the need for a molecular system for typing enteroviruses which is compatible with molecular diagnostic methods and which might eventually replace serotyping methods. Salient aspects of enterovirus genome structure and variability, which form the genetic basis of such a molecular typing system, are discussed. Possible applications of and approaches to molecular typing for enteroviruses are also discussed.

TABLE 1. Clinical manifestations of enterovirus infection

Syndrome	Commonly implicated serotype(s)	Reference(s)
Asymptomatic infection	All serotypes	223, 269
Paralytic poliomyelitis	PV 1 to PV 3	270, 288, 290, 330
	ENV 70	225, 226, 455
	ENV 71	75
	CAV 7	145, 454
Aseptic meningitis/ meningoencephalitis	PV, CBV, CAV, ECV	38, 87, 263, 378, 379
	ENV 71	10, 393
Acute myocarditis	CBV	142, 236
Bornholm disease (pleurodynia)	CBV	69, 145
Hand, foot, and mouth disease	CAV 16	367, 460
	ENV 71	56, 151
Herpangina	CAV, CBV, ECV	69, 145, 460
Exanthem	CAV, CBV	460
Acute hemorrhagic conjunctivitis	ENV 70	242
	CAV 24 (variant)	283
Neonatal multisystem disease	CBV, ECV	4–6, 150, 200
Nonspecific febrile/ respiratory illness	CAV, CBV, ECV	69, 86, 90

THE NEED FOR TYPING OF ENTEROVIRUSES

In uncomplicated situations, serotypic identification of an enterovirus isolate generally takes 1 to 2 weeks and usually has little impact on patient management. Thus, for clinical diagnosis it is often sufficient to establish a diagnosis of an "enterovirus infection" without further specifying the serotype (296, 381). However, in certain situations, serotypic identification is necessary or at least useful, and with the ability to type enteroviruses more rapidly by using molecular techniques, serotypic identification will possibly have greater impact on patient management.

Correct Identification of Poliovirus

The eradication of poliomyelitis and wild PV by the year 2000 is the aim of the WHO PEI (213, 468). Polio vaccination can then be stopped, but only when the eradication of wild PV has been documented convincingly. Therefore, identifying enterovirus strains as PV or nonpolio enteroviruses (NPEV) is extremely important. Since most PV infections are asymptomatic, demonstrating the absence of cases of paralytic poliomyelitis is an unreliable marker of eradication, particularly as the goal of eradication is approached (110), and it must be accompanied by virological monitoring. However, the extensive use of live PV (Sabin) vaccine results in fecal shedding of vaccine strain virus, which can survive sewage treatment (128) and persist in the environment for several months (389). The use of the Sabin vaccine also results in rare cases of vaccine-associated paralytic poliomyelitis (21, 315), probably caused by reversion to neurovirulence of vaccine strain virus as a result of a point mutation or recombination with wild PV (239). The ability to identify wild and vaccine-derived PV in clinical and environmental samples is therefore necessary to monitor the effectiveness of polio vaccination strategies (47, 286, 341, 423), to identify and monitor PV outbreaks (81, 167, 326, 411, 444), to diagnose cases of vaccine-associated paralytic poliomyelitis, and to exclude other enteroviruses as a cause of poliomyelitis-like disease (20, 75, 104, 114, 126, 143, 144, 225, 268, 454). A network of WHO-sponsored polio laboratories has been established to coordinate this task.

Studying Relationships between Enterovirus (Sub)type and Disease Manifestation

Although the disease spectra of the different serotypes overlap considerably, certain disease syndromes are caused by one or a few serotypes. The most striking example is of course the PV, which are the main cause of paralytic poliomyelitis. A typing method which gave a complete correlation between virus type and pathogenicity or virulence for other enterovirus syndromes would be difficult to achieve and would require a more thorough knowledge of the viral determinants of pathogenesis. However, some associations between enterovirus serotype and disease syndrome have been observed (145, 293, 416), and the use of sensitive molecular detection methods has recently proved of value in further delineating these associations. Thus, most cases of enteroviral meningitis have been found to be caused by certain CBV and ECV serotypes (38) whereas most cases of viral myocarditis are associated with CBV (196, 208, 312). Such knowledge may simplify virological diagnosis and identify enterovirus serotypes and diseases for which future effort should be directed toward understanding pathogenesis and designing therapeutic or preventive measures.

Identification of New Enterovirus Types or Variants

The ability to recognize previously unknown enterovirus types (39, 393) or strains (242) provides a basis for understanding changing patterns in the epidemiology and clinical manifestations of enterovirus infections. This has been of particular importance when the new type may cause poliomyelitis-like illness (10, 75, 225). Molecular detection methods are increasingly accepted as a means of identifying and characterizing previously unknown, noncultivable microorganisms and establishing their etiologic relationship to disease (reviewed in reference 116). This approach may also identify additional enterovirus types. One recent study identified novel enterovirus sequences, possibly representing previously unrecognized enterovirus types, in patients with chronic fatigue syndrome (77, 123).

Typing of Enteroviruses in Neonates and Immunodeficient Patients

Patients with hypo- or agammaglobulinemia are susceptible to chronic enterovirus infections which may cause persistent central nervous system infection, arthritis, dermatomyositis, or chronic enteritis (296), the most severe complication being a chronic meningoencephalitis (264). Some patients have been successfully treated with intravenous or intrathecal immunoglobulins with a high neutralizing-antibody titer against the infecting enterovirus serotype (106, 112, 264, 267). Immunoglobulin therapy has also been used in limited trials to treat neonates with fulminant enterovirus infections (5). This treatment was associated with a significant reduction in levels of viremia and viruria only when the immune globulin administered had a neutralization titer to the patient's isolate of at least 800. However, studies of immune globulin therapy in neonatal infections thus far have been too small to allow its widespread use to be recommended. Conversely, typing of enterovirus isolates has been used to exclude persistent enterovirus infection of the central nervous system in children with rapidly recurring episodes of aseptic meningitis due to repeated infection with different serotypes (9).

Epidemiological Monitoring

Enteroviruses have a worldwide distribution. Within a given geographic locality, some serotypes may be endemic, with little or only gradual change in the range of serotypes present from year to year. In temperate climates there is increased circulation in summer and early fall. In contrast, other serotypes may be introduced periodically, causing epidemics, with very few isolations reported in intervening years (294; reviewed in reference 295). Epidemiological surveillance plays a crucial role in the control of infectious diseases (356), and obtaining information on the serotypes of enteroviruses detected in clinical and environmental samples has formed an important part of surveillance programs (154, 170, 263, 416). Such knowledge may help in understanding changing patterns of enterovirus infection and disease association and may also facilitate the rapid diagnosis of enterovirus infections. This may reduce unnecessary hospitalization and other medical expenditure (11, 363), allow immune globulin batches with high titers to frequently circulating serotypes to be reserved for intravenous therapy of neonates or immunodeficient patients (5), and guide the formulation of antigens for serological diagnosis (11, 91).

CONVENTIONAL METHODS FOR DIAGNOSIS AND TYPING OF ENTEROVIRUSES AND THEIR LIMITATIONS

Enterovirus diagnosis is achieved by culturing virus from clinical specimens. When this is unsuccessful or not possible, demonstration of seroconversion, a fourfold or greater rise in type-specific virus-neutralizing antibody titers, or virus-specific immunoglobulin M (IgM) antibody in serum and/or cerebrospinal fluid (CSF) may provide evidence of infection. Methods for enterovirus diagnosis are detailed in references 138, 269, 296, and 381.

Virus Isolation

Isolation of an enterovirus from affected organs and associated body fluids (e.g., myocardium, pericardial fluid, or CSF) provides the strongest evidence of an enteroviral etiology. However, enteroviruses are rarely isolated from myocardium except in infants with fulminant infection, although the causal relationship between enteroviruses and acute myocarditis was originally established on this basis (reviewed in reference 142). Virus isolation from blood components is also useful and provides evidence of systemic infection (88, 345, 346). Detection of enterovirus in the alimentary tract (throat swabs, rectal swabs, or stool specimens) provides only circumstantial evidence of etiology, since viral shedding at these sites may occur in the absence of symptoms, particularly in infants and during epidemic seasons (223). However, stool is the most sensitive specimen for enterovirus detection, and stool culture is recommended for diagnosis of PV infections (470). Culturing enteroviruses from sewage and other environmental samples is of particular use for environmental monitoring and epidemiological studies.

Although virus isolation has been regarded as the "gold standard" for enterovirus identification, virus isolation procedures are only poorly standardized and virus isolation data may vary considerably between laboratories. Sensitivity is highly dependent on the type and quality of the specimen, the timing of specimen collection, and storage before arrival in the virus laboratory (138, 296). The choice of cell types used for enterovirus isolation is also important. No single cell line currently in use supports the growth of all known enterovirus serotypes (381). Therefore, to ensure maximal isolation efficiency, combinations of cell lines are generally used (68, 89). A presumptive identification of the enterovirus group can often be based upon growth of the isolate in different cell lines (193). Many enteroviruses grow well in primary monkey kidney cells. However, the supply of these cells is limited. CAV serotypes are difficult to grow in cell culture. Most have been propagated in RD cells (392, 459), but in practice isolation from clinical material is often unsuccessful (248). The use of suckling mice for isolation of CAV has been more successful (248), but because of practical difficulties this technique is rarely used. As a consequence, CAV infections are probably underdiagnosed and underrepresented in epidemiological surveys (416). PV may be selectively isolated in cultures of recombinant murine cells expressing the human PV receptor gene (169, 336). At present, there is no universal consensus on the optimal combination and number of cell lines required for enterovirus isolation, and different combinations are used in different laboratories.

Other factors affecting the efficiency of isolation are the physiological condition of the cells and whether blind passages are performed. Blind passages are necessary in some cases before a cytopathic effect in cell culture or paralysis in suckling mice becomes apparent. This may be due to the low initial titer of virus or the requirement for adaptation to growth in the isolation system (248). Under optimal conditions, a positive enterovirus isolation can be reported within a few days, but a presumptive diagnosis may require more than 14 days when dealing with difficult isolates and even longer for samples containing mixtures of viruses.

Enterovirus Serotyping

Following the isolation of an enterovirus, the serotypic identity can be determined by neutralization of infectivity with serotype-specific antisera. Typing by neutralization with reference antisera for all 66 serotypes individually is clearly impractical. To overcome this problem, equine type-specific hyperimmune sera have been mixed to give intersecting pools containing different combinations of individual antisera (138, 269, 391). Enterovirus isolates are incubated with each antiserum pool and then reinoculated onto susceptible cells. After incubation for several days, the neutralization pattern is recorded. From this, the enterovirus serotype can be inferred, since pools are designed so that neutralization patterns are distinct for each individual serotype. Finally, the suspected enterovirus type can be confirmed by neutralization with the type-specific single antiserum. The Lim Benyesh-Melnick (LBM) pool scheme consists of eight pools (designated A to H) containing antisera to 42 different enterovirus types (241, 271). Antibodies against 19 additional CAV serotypes in seven additional pools (designated J to P) can be used if identification with pools A to H cannot be achieved (272). Alternative intersecting pools have been developed at the National Institute of Public Health and the Environment (RIVM) in the Netherlands, which also allow identification of enterovirus types 68 to 71 (201). The RIVM pools are now used in the WHO polio laboratory network and are candidates to replace LBM pools.

There are several drawbacks to the use of intersecting pools for typing of enteroviruses. First, the method is time-consuming, labor-intensive, and costly. Second, the supply of antisera is limited, and WHO has advocated a conservative approach to the use of pools, which should not be used to type every clinical isolate (273). Third, the problem of "untypeable" enteroviruses is frequently encountered (296). There are several reasons for this. (i) Untypeable isolates may contain mixtures of enteroviruses. In the tropics, up to 50% of stool samples from infants may contain enteroviruses (224), and the frequency of mixed infections is presumably high. To resolve mixtures, isolates are purified by three serial terminal dilutions or plaque purifications. (ii) Not all enterovirus serotypes can be identified with intersecting pools (CAV 3, 11, 15, 17, and 24 and enterovirus types 68 to 71 cannot be typed using LBM pools). (iii) Enteroviruses sometimes form aggregates which can be neutralized only after treatment with sodium deoxycholate or chloroform or by ultrafiltration to dissociate the aggregates (202, 456). (iv) The isolates may be so-called prime strains. These are antigenic variants of recognized serotypes which are neutralized poorly or not at all by antiserum to the homologous prototype strain. However, antiserum raised against the prime strain will neutralize both prime and prototypic strains (269). This requires inoculation of experimental animals with the isolate to make an antiserum, which must then be tested for neutralization of the prototypes. (v) Isolates that are genuinely untypeable after excluding the above possible reasons for failure to type may represent a new or previously unrecognized enterovirus type. However, when first attempts at typing isolates using intersecting pools is unsuccessful, the additional labor and expense required to achieve serotypic identification are

usually considered prohibitive and such isolates remain untyped.

The standardization of enterovirus serotyping is problematic, and results derived from the LBM typing scheme may be influenced by the experience of laboratory personnel. This is illustrated by results of a small-scale quality control scheme on enterovirus isolation. A panel of 10 samples was sent out to 17 laboratories. None of the 11 participating laboratories that performed virus isolation and serotyping reported correct results for all the samples (449).

Because of the practical problems and limited clinical relevance of enterovirus serotyping, the number of laboratories offering this service has steadily decreased. At present, typing is performed mainly in research and reference laboratories. Attempts have been made to shorten the time required to identify and type enterovirus isolates by detecting the virus by enzyme immunoassays with type-specific antisera (480), by using immunoelectron microscopy with polyvalent and type-specific antisera (237, 310), or by using early detection of enterovirus antigens in cell culture by indirect immunofluorescence or enzyme immunoassay with monoclonal antibodies to group-reactive (218, 433, 483, 484) or type-specific (27, 79, 218) determinants. Type-specific monoclonal antibodies have been found useful for identification of CBV isolates, including prime strains (229). Although these methods can provide a positive identification more rapidly than neutralization assays, none are in widespread use.

Serotyping is, however, used by the WHO polio laboratory network for the serotypic identification of PV and intratypic differentiation of vaccine or vaccine-derived strains from wild PV strains (reviewed in reference 450). Intratypic differentiation is based on the detection of antigenic differences between vaccine strains and currently circulating wild PV strains, and it may be achieved by neutralization of infectivity with cross-absorbed polyclonal antisera specific for either Sabin vaccine strains or common wild strains (327, 451, 469) or panels of vaccine-derived and/or wild PV strain-specific monoclonal antibodies (85, 101, 113, 189, 327). The neutralization test involving polyclonal antisera has now been replaced by an enzyme immunoassay (131, 327, 443), which allows a more conservative use of antisera.

Serological Diagnosis

Serological diagnosis of enterovirus infections is complicated by the large number of serotypes; the occurrence of anamnestic, heterotypic antibody responses; and the lack of a uniformly cross-reactive enterovirus group antigen. The requirement for appropriately timed paired sera to demonstrate a diagnostically significant rise in antibody titer further limits the utility of enterovirus serologic testing. The demonstration of enterovirus-specific IgM antibody in a single serum, indicative of a current antigenic stimulus, provides evidence of recent infection (11, 32, 33, 45, 59, 96, 111, 119, 256, 262, 388, 430), but results must be interpreted with caution, since background levels of IgM in the general population may be high (277), particularly during epidemic seasons. In addition, the persistence of IgM responses for some time beyond the end of acute infection has been observed in patients with chronic disease (305). Thus, while detection of IgM in serum may be more sensitive than virus isolation in detecting enterovirus infection (32, 96), this provides only circumstantial evidence of an etiologic role in concurrent symptoms. However, detection of virus-specific IgM in CSF is more likely to indicate a causal relationship to CNS symptoms and is more sensitive than isolation of PV from stool for confirming a diagnosis of paralytic poliomyelitis (311, 369). Determination of virus-specific IgM in serum has proved useful in monitoring a recent PV outbreak in the Netherlands (326) and in defining the epidemiology of NPEV outbreaks (11, 134).

ENTEROVIRUS DETECTION BY PCR

Genetic similarities among enterovirus serotypes allow the use of generic cDNA hybridization probes derived from one enterovirus serotype to detect genomes of a wide range of enteroviruses (49, 181, 198, 434; reviewed in reference 377). Hybridization technology has also been successfully used to detect enterovirus RNA in clinical material from patients with suspected enterovirus infection (16, 17, 49, 84, 107, 197). Also, the use of in situ hybridization in particular may provide useful information on the cellular distribution of virus infection and its relationship to inflammatory or necrotic lesions (83, 219, 220). However, nucleic acid hybridization technology is laborious and is not always sufficiently sensitive for diagnosis, for example, in the detection of enterovirus RNA in CSF (reviewed in reference 377). PCR has therefore been explored as a straightforward yet sensitive means of enterovirus genome detection. Comparison of published enterovirus genome sequences has allowed highly conserved sequences to be identified. These have been exploited as primer recognition sequences for reverse transcriptase PCR (RT-PCR) assays capable of detecting most or all enteroviruses, including those which cannot readily be propagated in cell culture (63, 178, 196, 376, 494) and untypeable isolates (394). When carefully optimized, these assays are at least as sensitive as cell culture systems for the detection of enteroviruses in clinical specimens (2, 7, 57, 132, 133, 153, 192, 302, 313, 324, 380, 382, 390, 427, 478, 494) and environmental samples (1, 98, 228, 348, 436, 437). Results can be obtained in as little as 5 h (382) and may therefore have a greater impact on clinical decision-making. It is also possible to detect enterovirus RNA by RT-PCR in specimens from which infectious virus is never or only rarely recovered by culture, such as myocardial tissue from patients with acute myocarditis or dilated cardiomyopathy (196, 208, 258, 395) and in formalin-fixed, paraffin-embedded tissue (161, 240, 304, 312, 358, 403, 465).

RT-PCR will therefore assume a major role in the laboratory diagnosis of enterovirus infections. An enterovirus PCR test kit for the diagnosis of enteroviral meningitis, now commercially available from Roche Molecular Systems, will assist in the transition to PCR-based diagnosis (199, 382, 478). This test is based on single-step reverse transcription and amplification of enterovirus RNA, and it incorporates enzymatic elimination of amplicon contamination, thus harmonizing and simplifying laboratory procedures and reducing the risk of false positivity due to carryover of previously amplified nucleic acid. However, quality assurance procedures will be required to ensure test sensitivity, specificity, and reliability; experience with PCR assays for other viruses has revealed considerable interlaboratory variation in sensitivity, specificity, and reliability, which is partially but not completely alleviated by the use of standardized methods or commercial assays (46, 93, 185, 351, 442, 485, 486).

A limitation of most PCR methods described thus far for the diagnosis of enterovirus infections is the inability to perform serotyping or other subclassification of enteroviruses. When such information is required, it may therefore be necessary to supplement PCR detection with traditional culture methods to provide a virus isolate for typing purposes (109). However, due to the greater sensitivity of PCR, cell culture in many cases will not yield a virus isolate. As an alternative approach, nucleotide

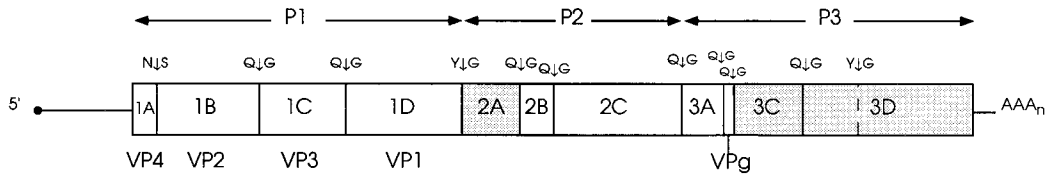

FIG. 1. Genetic organization of poliovirus type 1 (Mahoney), the type member of genus *Enterovirus* of *Picornaviridae*. The polyprotein encoded by the single open reading frame is shown as an elongated rectangle, the 5′ and 3′ noncoding regions are shown as lines, and the genome-linked protein (VPg) is indicated by a black circle. Cleavage sites between individual viral proteins are shown above the genome at appropriate locations; these proteins are described within the rectangle according to the L434 nomenclature (383a); the capsid proteins 1AB, 1A, 1B, 1C, and 1D are commonly referred to as VP0, VP4, VP2, VP3, and VP1, respectively. The proteinases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ are represented by shaded boxes. The structural protein precursor P1 and the nonstructural protein precursors P2 and P3 are indicated above the polyprotein. Reprinted from reference 160 with permission of the publisher.

sequence analysis of PCR products may allow the typing of viruses detected only by PCR (reviewed in reference 78). A few studies have compared the nucleotide sequence of enterovirus PCR products with published enterovirus sequences to type or classify enteroviruses in the absence of a virus isolate (19, 208, 303, 304, 312). Other methods of assessing sequence differences have also been described, including restriction fragment length polymorphism (RFLP) analysis of PCR products (PCR-RFLP) (24, 196, 313, 396), hybridization with type-specific probes (63, 94, 421), or single-strand conformation polymorphism (SSCP) analysis of PCR products (PCR-SSCP) (121). A molecular typing system based on such methods could minimize the requirement for isolating enteroviruses in culture for most common diagnostic and epidemiological applications and may provide additional biological information which could not have been obtained by serotyping methods.

GENETIC BASIS OF MOLECULAR TYPING FOR ENTEROVIRUSES

Development of a molecular typing system for enteroviruses requires an understanding of the structure and function of the enterovirus genome and a knowledge of the variability of genome sequences among enteroviruses.

Enterovirus Genome Structure

The enterovirus genome is a single-stranded RNA molecule, approximately 7,500 nucleotides long, of positive polarity (Fig. 1) (reviewed in references 160 and 383). An approximately 750-nucleotide 5′ untranslated region (5′ UTR) is followed by a long open reading frame coding for an approximately 2,100-amino-acid polyprotein. This is followed by a short 3′ untranslated region (3′ UTR) and a poly(A) tail.

5′ UTR. Several important functions have been mapped to the 5′ UTR. For PV, the first 100 nucleotides (approximately) play a role in viral replication (13). Enteroviruses use internal initiation of translation rather than the ribosome-scanning model proposed for cellular mRNAs (333). This internal initiation has been shown to require a large portion (nucleotides 130 to about 600) of the PV 5′ UTR (40, 152), but the exact ribosome-binding sequence is not known. Point mutations in the 5′ UTR have been shown to affect virulence (55, 204, 278, 299, 438), temperature sensitivity, and plaque morphology (354).

Open reading frame. The open reading frame following the 5′ UTR is translated into a polyprotein which is co- and post-translationally cleaved to give four structural proteins (VP4, VP2, VP3, and VP1), which form the viral capsid, and seven nonstructural proteins (217). VP1 to VP3 are partially exposed on the virion surface, while VP4 is completely internalized in infectious virions. Three or four neutralization determinants have been identified for each PV serotype by using monoclonal antibody neutralization escape mutants, and they have been mapped to VP1, VP2, and/or VP3 (reviewed in reference 278). Neutralization determinants have also been identified on VP1 for CBV 3 (149) and CBV 4 (266, 359). Determinants of attenuation of virulence (52, 278, 355), virion thermostability (278), altered host range (82), in vitro cell tropism (245), persistent infection (53, 331), and plaque morphology (354, 488) have also been mapped to the capsid-encoding region.

Some functions of the nonstructural proteins and their precursor forms are known. Protein 2A is one of the viral proteinases that cleaves the polyprotein in *trans* between proteins VP1 and 2A and frees the capsid protein precursor from the rest of the polyprotein (432). Proteinase 2A has also been shown to participate in host cell shutoff by indirectly inducing cleavage of the cellular p220 protein, which is an important factor in cap-dependent initiation of translation (412). The specific functions of 2B and 2C are not known, although protein 2C and its precursor form 2BC have been found in the replication complex of PV (41), and protein 2C has a helicase activity. Protein 3AB is a precursor of 3B, the small polypeptide covalently linked to the 5′ UTR of picornavirus RNA molecules (115). Protein 3C is the second viral proteinase which does most of the polyprotein processing (155, 156), while 3D is the RNA-dependent RNA polymerase (447).

3′ UTR. The coding region is followed by a 70- to 100-nucleotide 3′ UTR. This region is important in the initiation of the minus-strand RNA synthesis, but no specific sequences have been identified for polymerase binding. Several secondary and tertiary structures (pseudoknots) have been proposed for this region (182, 342) and supported by biochemical studies (186, 335). A genomic poly(A) tail with an average length of 75 nucleotides follows the 3′ UTR of all enteroviruses (18).

Genetic Relationships among Human Enteroviruses

Complete genome sequences for a number of human enterovirus serotypes have been published (51, 60, 92, 139, 173, 174, 179, 182, 190, 217, 221, 232, 246, 317, 337, 339, 353, 384, 413, 414, 419, 431, 487, 493) (Table 2). Partial sequence data are available for the majority of the remaining serotypes (23, 103, 105, 130, 177, 339, 342, 349, 372, 461) (Table 2). While sequence comparisons partially support the classical subgrouping of enterovirus serotypes into PV, CAV, CBV, and ECV, in many cases genetic relationships do not correlate with this division. Nucleotide sequence comparison of the several genomic regions indicates that while serotypes within the PV and CBV groups are genetically related, CAV and ECV are genetically diverse subgroups. ECV types 22 and 23 differ significantly from the rest of the enteroviruses genetically (179, 415). Although these viruses are still currently classified as enteroviruses (279), the International Committee on Taxon-

TABLE 2. Enterovirus sequences used in Fig. 2 and 3

Enterovirus strain description	Abbreviation	GenBank accession no.	Reference
CAV 1/T.T	CAV 1	X87584	349
CAV 2/Fleetwood	CAV 2	L28146	349
CAV 3/J.Ol.	CAV 3	X87586	349
CAV 5/G.S.	CAV 5	X87588	349
CAV 7/AB-IV	CAV 7	X87589	349
CAV 8/C.D.	CAV 8	X87590	349
CAV 9/Griggs	CAV 9	D00627	60
CAV 10/M.K.	CAV 10	X87591	349
CAV 11/Belgium 1	CAV 11	X87592	349
CAV 13/Flores	CAV 13	X87594	349
CAV 14/G-14	CAV 14	X87595	349
CAV 16/G-10	CAV 16	U05876	339
CAV 18/G-13	CAV 18	X87598	349
CAV 20/IH Pool 35	CAV 20	X87600	349
CAV 20b/Cecil P2647	CAV 20b	X87602	349
CAV 21/Coe	CAV 21	D00538	174
CAV 24/EH 24/70	CAV 24	D90457	419
CBV 1/Japan	CBV 1	M16560	182
CBV 3/Nancy	CBV 3	M33854	221
CBV 4/JVB	CBV 4	X05690	190
CBV 5/1954/UK/85	CBV 5	X67706	487
ECV 1/Farouk	ECV 1	L76395	342
ECV 6/Finland/88	ECV 6/FIN/88	L76399	342
ECV 7/Wallace	ECV 7	L76401	342
ECV 7/Finland/87	ECV 7/FIN/87	L76400	342
ECV 11/Gregory	ECV 11	X80059	92
ECV 12/Travis	ECV 12	X79047	232
ECV 27/Bacon	ECV 27	L76396	342
ECV 30/Finland/85	ECV 30/FIN/85	L76397	342
		L76398	342
ENV 68/Fermon	ENV 68	X87604	349
ENV 69/Toluca-1	ENV 69	X87605	349
ENV 70/J670/71	ENV 70	D00820	384
ENV 71/MS7423/87	ENV 71/87	U22522	51
PV 1/Mahoney	PV 1m	V01148	353
PV 1/1400/Mexico/80	PV 1/MEX/80	L76404	342
PV 1/15/Hong Kong/81	PV 1/HK/81	L76402	342
PV 1/NE-459/Spain/82	PV 1/SPA/82	L76409	342
PV 2/Lansing	PV 21	M12197	233
PV 2/Sabin	PV 2s	X00595	431
PV 2/299/USA-Ca/52	PV 2/USA/52	L76412	342
PV 2/364/India/56	PV 2/IND/56	L76393	342
PV 2/LS2575/Kuwait/80	PV 2/KUW/80	L76403	342
PV 3/Leon/37	PV 31	K01392	413
PV 3/23127/Finland/84	PV 3f	X04468	173
PV 3/Saukett E	PV 3sa-E	L23846	175
PV 3/1620/Netherlands/58	PV 3/NET/58	L76406	342
PV 3/17206/Netherlands/70	PV 3/NET/70	L76407	342
PV 3/21267/Marocco/77	PV 3/MAR/77	L76405	342
PV 3/2141/Switzerland/80	PV 3/SWI/80	L76410	342
PV 3/20800/Turkey/81	PV 3/TUR/81	L76411	342
PV 3/F29/Singapore/86	PV 3/SIN/86	L76408	342
PV 3/30865/Bolivia/86	PV 3/BOL/86	L76394	342
PV 3/50/USSR/87	PV 3/USSR/87	L76413	342

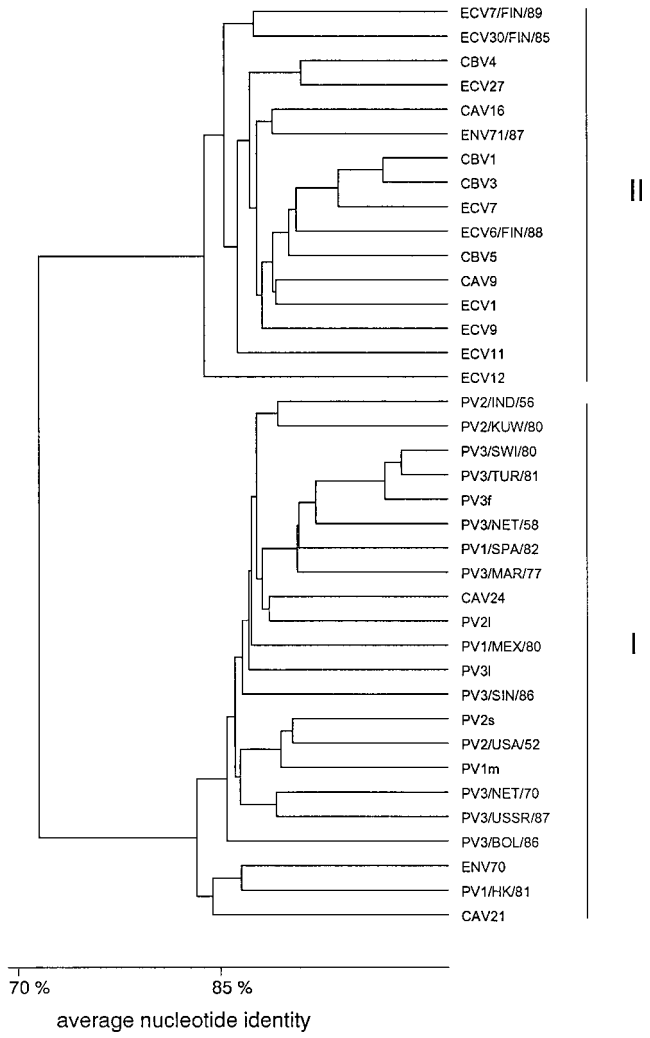

FIG. 2. Dendrogram based on the nucleotide identity of the 5′ UTR. Sequences used for comparison are given in Table 2.

omy of Viruses Picornavirus Study Group has recently proposed that they be reclassified into a separate genus, provisionally designated human orphanovirus (HOV) types 1 and 2, respectively. The remaining ECV serotypes are genetically related to the CBV group (177).

The UTRs are the most highly conserved parts of the enterovirus genome. For example, the three Sabin strains of PV have 85% nucleotide identity in the 5′ UTR and approximately 90% identity in the 3′ UTR (413, 431). Toyoda et al. (431) showed that the 5′ UTR could be divided into conserved (nucleotides 1 to 650) and hypervariable (nucleotides 651 to 750) regions according to the variation seen among the three Sabin strains. Three computer-predicted secondary structures have been proposed for the conserved part of the 5′ UTR, and this model is supported by biochemical studies (334, 366, 410). The nucleotide sequence variation seen in this region of all sequenced enteroviruses shows mainly compensatory base changes that support the proposed stem-loop structures (105, 177, 340, 342, 372). Particularly between nucleotides 440 and 600 of the enterovirus genome, there are a number of short nucleotide stretches showing almost perfect conservation among all enterovirus groups, as well as in some cases the genetically more distant rhinoviruses. These have been exploited as primer and probe recognition sequences for broadly reactive diagnostic PCR assays. Comparison of the 5′ UTR of human enteroviruses reveals two clusters with a minimum nucleotide identity of 77% within each cluster (Fig. 2): PV, CAV 21, CAV 24, and ENV 70 form one cluster (I); and all CBV serotypes, CAV 9, CAV 16, ENV 71, ECV 11, ECV 12, and all other partially sequenced ECV serotypes form a second cluster (II) (177, 180, 342).

While the need to maintain some identical nucleotide stretches and the secondary structure seems to restrict the

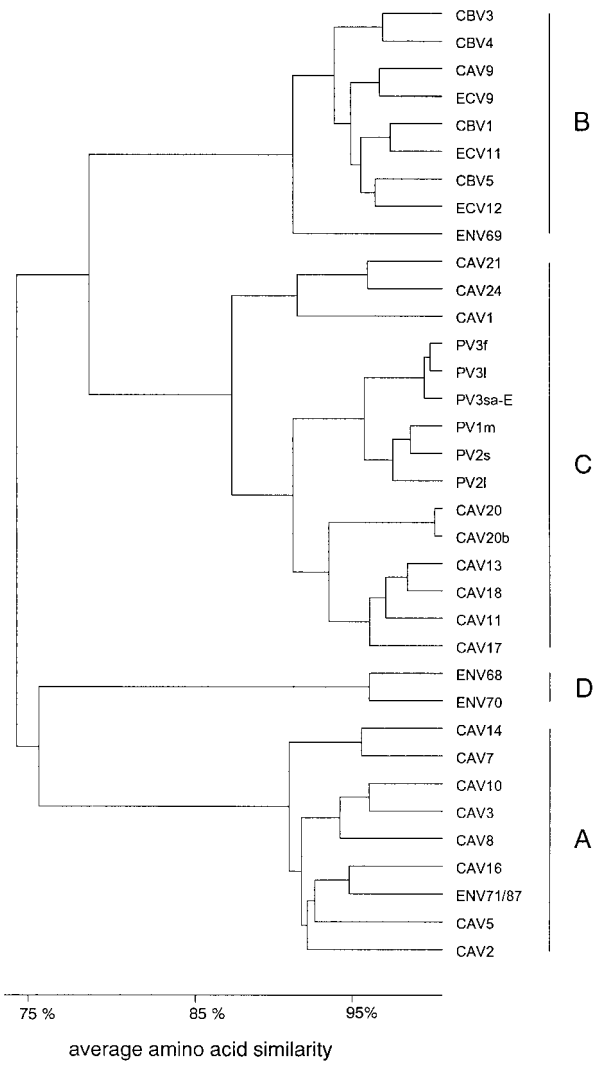

FIG. 3. Genetic relationships among human enteroviruses. Dendrogram based on amino acid similarity of a 70-amino-acid sequence in the junction of VP4 and VP2. Sequences used for comparison are given in Table 2.

variation in the 5′ UTR, the functional polypeptide structure places the main constraint on variation in the coding region, although higher-order structural requirements may also restrict variation. Most of the variation seen in the protein coding region is silent; i.e., the predicted amino acid sequence is not altered. Each of the two clusters based on sequence similarity in the 5′ UTR can be further subdivided into two separate clusters based on nucleotide and amino acid sequence similarity in the coding region. When the partial nucleotide sequence of VP2 protein is compared, CAV 2, CAV 3, CAV 5, CAV 7, CAV 8, CAV 10, CAV 14, CAV 16, and ENV 71 form one cluster (A); all CBV serotypes, CAV 9, ECV 11, ECV 12, and ENV 69 form another cluster (B); all PV serotypes, CAV 1, CAV 11, CAV 13, CAV 17, CAV 18, CAV 20, CAV 21, and CAV 24 form the third cluster (C); and ENV 68 and ENV 70 form the fourth cluster (D) (349) (Fig. 3). The clustering of enteroviruses is the same regardless of which protein is used in the comparison, provided that complete protein-coding sequences are used in the analysis (342). Sequence variation is highest in VP1, which codes for the major antigenic sites and the most type-specific neutralization determinants. The amino acid similarity is at least 71% within a cluster and ranges from 53 to 68% between clusters in the capsid-coding region (180, 342).

The nonstructural proteins show considerably less variation than the structural proteins do. While variation in the capsid proteins may be advantageous, enabling the virus to escape from host immune responses, variation in the nonstructural proteins, many of which are enzymes, might be deleterious. In general, amino acid similarity in nonstructural proteins is high, usually up to 90% within a cluster, being highest in the 3D protein, which is the RNA polymerase.

Clustering of the 3′ UTR is the same as that seen in the coding region, with a minimum nucleotide identity of 76% within a cluster (342). The most prominent variation between the clusters is its length: PV-like viruses have the shortest 3′ UTR (69 to 72 nucleotides), while CBV-like viruses have the longest (102 to 106 nucleotides) and CAV 16-like viruses and ENV 70 have an intermediate-length 3′ UTR (82 to 86 nucleotides). A similar secondary structure can be predicted for all the members in each cluster, although there are minor differences between clusters. Two stem-loop structures can be predicted for ENV 70 and the PV-like viruses, and three stem-loop structures can be predicted for the CBV- and CAV 16-like viruses (335, 342). It seems that the need to maintain some identical nucleotides as well as the secondary structure limits the variation in the 3′ UTR.

Intratypic Genetic Variation in Enteroviruses

In common with many other RNA viruses, enteroviruses exhibit a high degree of intratypic sequence heterogeneity, resulting from the relatively high rate of nucleotide misincorporation during viral RNA replication both in vitro and in vivo (163, 164, 215, 318, 457). The sequence variation with a single serotype is, in many genomic regions, often as high as that observed between serotypes (105, 216, 227, 340, 495) (Table 3). The structural proteins, especially the antigenic sites, show greatest intratypic variation. This has been shown both by nucleotide sequence analysis (215) and by demonstration of the differential reactivity of isolates with panels of neutralizing monoclonal antibodies (85, 171, 215, 332, 344, 458). On comparing VP1 coding sequences of epidemiologically unrelated isolates of a single serotype, up to 25% nucleotide divergence may be observed, corresponding to up to 17% divergence in the amino acid sequence (Table 3). Intratypic sequence variation is observed to a lesser extent in the untranslated and nonstructural genes (Table 3).

TABLE 3. Intratypic nucleotide and amino acid sequence diversity of selected regions of enterovirus genomes

Serotype	% Maximum nucleotide (amino acid) sequence divergence			Reference(s)
	5′ UTR	Capsid (VP1)	NS[a] proteins	
PV 1		22.6 (8)		365
ECV 30	10–30			103, 105
CBV 1	11		25 (5)	105, 495
CBV 3	11			125
CBV 4		20	12	172
CBV 5		16		227
CAV 9		24.4 (17)		61
CAV 24v			12 (4.4)	183
ENV 70		11 (3.9)		422, 475

[a] NS, nonstructural.

POSSIBLE APPLICATIONS OF MOLECULAR TYPING OF ENTEROVIRUSES

A molecular typing system for enteroviruses will provide a valuable alternative to the existing serological methods for identifying enterovirus types and for epidemiological monitoring. Molecular typing may also prove useful for studying other properties of enteroviruses which cannot be addressed by traditional typing methods.

Genotypic Classification of Enteroviruses

Several virus groups that have not been amenable to serological investigation due to difficulties in laboratory propagation have been classified according to molecular genetic criteria. These include the human papillomaviruses (102), the human polyomavirus JC (22, 148, 479), hepatitis C viruses (HCV) (320, 321, 407, 408, 417, 418), and hepatitis D viruses (473), and subtypes of human immunodeficiency virus type 1 (HIV-1) (463). Genotypic classification has proved invaluable for epidemiological investigation and for studying differences in biological phenotype such as virulence and antiviral susceptibility.

HCV provides a useful analogy for molecular typing systems. HCV has a positive-sense RNA genome of approximately 10,000 kb and was discovered by molecular cloning techniques. Based on sequence information, a growing number of distinct HCV genotypes and subtypes have been described. At present, 6 major genotypes and more than 50 subtypes have been differentiated (407, 408, 417, 418, 429). HCV genotyping is useful for epidemiological analysis and for prediction of the likely response of chronic HCV infection to alpha interferon therapy (reviewed in reference 406). Identification of HCV genotypes is thus of clinical importance. While nucleotide sequencing remains the gold standard for genotype determination, other differentiating molecular methods used include PCR-RFLP analysis (95, 265, 309), genotype-specific PCR (64, 320–322), and group-specific PCR followed by genotype-specific hybridization or reverse hybridization (line probe assay) (357, 417, 418, 428, 452). Some methods originally used genomic regions now known to have too little variability to differentiate all types and subtypes. However, difficulty in accommodating the increasing number of newly characterized genotypes and subtypes presents problems for all these typing methods (418).

As described above, human enteroviruses can be classified into two major phylogenetic groups based on 5′ UTR sequence similarity and into four major groups based on similarity in the coding region or the 3′ UTR. Recently, a PCR assay combined with amplicon sequencing which allows enteroviruses to be assigned to one of the four clusters has been described (19). It has been suggested that this should form the basis of an alternative genetic classification of enteroviruses (19, 180, 349). A region covering part of the 5′ UTR, the VP4, and the amino terminus of the VP2 protein was amplified, and sequences obtained from the VP2-coding region were analyzed. This allowed the correct genetic classification of virus isolates in clinical samples, although some PCR products derived directly from clinical samples did not yield unambiguous sequence data. Electrophoretic sizing of 3′ UTR-derived PCR products may provide an alternative means for differentiating the major enterovirus subgroups.

Classification of enteroviruses based on phylogenetic relationships will be useful in studying viral genetics, taxonomy, evolution, and epidemiology. However, phylogenetic classification may not correlate consistently with disease phenotype. For example, NPEV types that cause paralytic disease do not show a close phylogenetic relationship to PV (51). Also, a phylogenetic classification would not provide information on serotypic identity. For these reasons, such a classification may be of limited clinical value. In no other case has conventional virus taxonomy based on serotypic classification been replaced by a genotypic classification, although alternative genotypic classification methods have been described for adenoviruses (reviewed in reference 401). In this situation, however, an initial genotypic classification of adenoviruses may simplify the subsequent task of serotypic identification by limiting the number of neutralization tests required (210). Similarly, an initial genotypic classification of enteroviruses may assist in subsequent identification of enterovirus serotypes or other biological phenotypes discussed below.

Identification of Enterovirus Serotypes

Despite the large number of enterovirus serotypes and the high degree of antigenic variation, the serotypes appear to be stable, probably reflecting the conservation of epitopes that are critical for capsid structure or function (reviewed in reference 260). It should therefore be possible to identify the genetic determinants of epitopes that define each individual serotype. The argument in favor of retaining a serotypic classification of enteroviruses is greater than simply one of familiarity. The concept of serotype is an important biological phenotype which is closely related to immunity and, in some cases, to clinical presentation. Serotype-specific identification is therefore of paramount importance in PV surveillance, and this would also be true of any other serotype for which vaccination subsequently becomes available. The potential need for serotypic identification in the context of the infected neonate or immunodeficient patient has already been discussed.

The serotype of an enterovirus is defined by neutralizing epitopes generally located on surface-exposed loops of the virus capsid proteins, particularly on VP1 but also on VP2 and VP3 (260, 280). Several PV neutralization determinants are conformation dependent and in some cases are formed by amino acid residues from different capsid proteins in close proximity on the virion surface. Therefore, it would be difficult to predict the precise neutralization characteristics of an enterovirus from nucleotide sequence data. Most linear epitopes identified to date are cross-reactive and nonneutralizing (58, 168, 386, 387). However, important neutralization determinants formed from uninterrupted colinear amino acid residues have been identified for PV (278, 280), CBV 3 (149), and CBV 4 (266, 359), and it may be possible to identify "signature" neutralizing epitopes from the nucleotide sequence of the relevant genomic region which permit serotypic identification. It would be necessary to identify epitopes conserved among a wide range of field isolates for the development of molecular typing reagents. Capsid protein residues which do not form part of neutralization epitopes may nonetheless influence antigenicity by stabilizing the local tertiary structure of the antigenic site (328), and there may be sufficient constraint on their genetic variability for such capsid regions to be useful in serotypic identification. Relatively highly conserved sequences have been identified in VP4- and VP2-encoding regions, and these have been used to design broadly reactive PCR assays from which information on the serotype or genetic group could be obtained by analysis of the intervening sequence (130, 177, 211, 238, 303, 312, 324, 349, 386, 422, 477). However, these assays are less broadly reactive than those which target the 5′ UTR. Because of sequence variability in capsid-encoding regions, it is unlikely that a single primer pair will allow the amplification of all serotypes, and the use of degenerate or multiplex primers will probably be necessary. Recent improve-

ments in PCR technology make this approach feasible (42, 404). Selection of primers that target relatively highly conserved capsid regions flanking more variable regions which distinguish individual serotypes may allow the use of several multiplex primer pairs for the amplification of a wide range of enteroviruses. Use of an initial genotyping assay, as discussed above, may reduce the number of multiplex primers required for subsequent serotypic identification, which could be achieved by molecular analysis of PCR products by methods described above. In view of the large number of enterovirus serotypes, a subsequent line probe assay analogous to that developed for HCV genotyping (417, 418) or microchip analysis as used to study resistance to HIV protease inhibitors (231) would be practically advantageous. In these assays, the virus type is identified in a single post-PCR reverse hybridization with type-specific oligonucleotides immobilized in lines on a membrane support or in high-density arrays on a microchip.

Other genomic regions that do not encode serotype-specific determinants are more conserved across serotypes and may evolve or undergo recombination (122, 127, 281) independently of virus serotype. It is therefore unlikely that serotypic identification of field enterovirus isolates could be based on the nucleotide or deduced amino acid sequence of nonstructural genomic regions. Some have attempted to identify enterovirus serotypes on this basis (reviewed in reference 306), for example, by analyzing PCR products generated with enterovirus group-reactive primers by high-stringency hybridization (63), PCR-RFLP analysis (196, 313), PCR-SSCP analysis (121), or nucleotide sequence determination (304, 312). However, the serotypic sensitivity and specificity of these discriminatory mechanisms have not been fully evaluated by analysis of a large number and wide range of serologically characterized field enterovirus strains. PCR-RFLP has been found useful only for PV and CBV, in part due to lack of sequence data for most ECV and CAV serotypes (196). Identification of all 66 human enterovirus serotypes by PCR-RFLP is unlikely to be practical: one suggested restriction enzyme digestion flow chart requires a total of nine different restriction enzymes and allows the identification of only 15 serotypes (196). Another drawback of RFLP analysis is that single point mutations within the recognition sequence may give rise to unclassifiable results. The use of PCR-SSCP for the analysis of enterovirus genotypes by using PCR products from the 5′ UTR has also been described (121). Different electrophoretic profiles were reported for 14 enterovirus serotypes investigated. However, this method is also very susceptible to minor sequence variation. Furthermore, SSCP analysis requires additional equipment and is time-consuming.

Attempts have been made to devise genetic typing methods for numerous other virus groups that have been classified by serological methods. Serological relationships among virus groups classified by genetic methods have also been studied. Genetic typing methods that show good correlation with serological classification methods have been reported for influenza virus types (76, 471) and influenza virus type A hemagglutinin and neuraminidase subtypes (471), some but not all hepatitis B virus subtypes (108, 314, 323), human herpesvirus types (214, 261, 426, 445), human T-cell lymphotropic viruses (439, 441), HIV types (405, 440), human polyomavirus BK groups (191), and serotypes of rotaviruses (135, 140), adenoviruses (ocular serotypes) (385), HCV (409, 428, 446, 490), dengue viruses (235, 298, 397, 398), astroviruses (316), hantaviruses (350), California group bunyaviruses (43), and foot-and-mouth disease viruses (250, 368, 448). Only partial correlation has been observed between HIV-1 subtypes and gp120 serotypes, but no correlation between HIV-1 subtype and neutralization type was observed (65, 230, 292), presumably reflecting a dependence of neutralization determinants on conformational structures which cannot be predicted from primary nucleotide or deduced amino acid sequences. Partial correlation was also observed for antigenic types of Norwalk and related small, round, structured viruses (14). A method for genetic typing of human rhinoviruses has been described (297), but this was not designed to correlate closely with serotypic classification.

The extent to which these genetic typing methods mirror serotyping methods depends on the degree of phylogenetic similarity between serotypes in the genomic region targeted by genetic typing methods (which affects typing specificity) and the extent of genetic variation within serotypes (which affects sensitivity), particularly with field strains. There is a high degree of phylogenetic similarity among enterovirus serotypes in most genomic regions, while the regions showing greatest divergence among serotypes also show greatest variability within serotypes. The human enterovirus group is considerably larger than most of the above virus groups, and the development of a molecular typing system which identifies all, or even the most important, serotypes presents a formidable challenge.

Intratypic Differentiation of Poliovirus

Currently used methods to differentiate vaccine or vaccine-derived strains from wild PV strains rely on genetic or antigenic differences between currently circulating wild PV strains and those from which the oral Sabin vaccine strains were derived, as a result of continuous genetic drift. A number of molecular methods have been developed, including oligonucleotide mapping of RNase T1-digested viral RNA by two-dimensional gel electrophoresis (318), hybridization of viral RNA with vaccine- or wild-strain-specific synthetic oligonucleotide probes (94) or riboprobes (97), PCR amplification of viral RNA with primers specific for Sabin vaccine strains (476) or current wild strains (307, 308, 477), and PCR with enterovirus group-reactive primers for PCR amplification followed by RFLP analysis (24, 122, 396), high-stringency hybridization with vaccine-strain-derived PCR product probes (421), or nucleotide sequence determination (365). Chumakov and coworkers (73, 74, 361, 362, 420) have also described a PCR-RFLP assay to identify and quantify the presence of neurovirulent revertants of Sabin vaccine strain virus in vaccine lots as a means of ensuring vaccine safety, while Guillot et al. (147) have described a site-specific PCR to detect the excretion of such revertants by recipients of Sabin polio vaccine.

Molecular methods for intratypic differentiation of PV currently used by the WHO polio laboratory network include RNA probe hybridization, strain-specific PCR, and PCR-RFLP analysis. In a recent study comparing the reliability of these and currently used serological methods (enzyme immunoassay with cross-absorbed polyclonal antisera and virus neutralization with strain-specific monoclonal antibodies) in five WHO laboratories with a coded panel of wild and vaccine strain viruses, all the methods gave correct results in 91.9 to 97.8% of tests (443). The RNA probe hybridization assay and enzyme immunoassay with polyclonal antisera performed best, but most discrepancies were obtained by laboratories using techniques that had only recently been introduced. However, only the enzyme immunoassay was able to detect and confirm the presence of both Sabin-like and wild non-Sabin-like viruses in samples containing mixtures of vaccine and wild PV. It was recommended that at least two methods of intratypic differentiation based on fundamentally different principles (e.g., antigenic differences or sequence differences) be used, with nucle-

otide sequence determination being used to resolve discrepant results.

Although these methods have proved successful, their use has thus far been restricted to analysis of PV which have first been isolated in cell culture and characterized as PV by standard methods. The use of molecular biological methods for both detection and (if possible simultaneous) intratypic differentiation of PV in clinical and environmental samples may lead to further improvement in the streamlining, standardization, and sensitivity of test procedures. This would require an assay that detects all PV types and strains, but not other enteroviruses, to identify PV-containing samples for subsequent intratypic differentiation. Given the extent of genetic variation among wild PV strains and their phylogenetic similarity to some NPEV strains, designing such a PV-specific assay presents theoretical difficulties. However, a number of promising methods have been described. Using primers located in the 5′ UTR designed to selectively amplify PV, Abraham et al. (3) reported a sensitivity of 100% on testing 81 PV isolates and a specificity of 96% on testing 50 NPEV isolates, while Egger et al. (109) reported a sensitivity of 100% on testing 81 PV isolates when two primer pairs located in the P2-P3 region were used and a specificity of 98% on testing 45 NPEV isolates. Using primers in the VP1-2A region, either alone or in combination with general enterovirus-reactive primers, Chezzi (66) reported 100% sensitivity and specificity on testing 125 PV and 38 NPEV isolates. Finally, Kilpatrick et al. (211) used primers containing mixed-base or deoxyinosine residues designed to recognize VP1-encoding regions unique to PV and successfully detected all of 158 wild or vaccine-related PV strains but none of 49 NPEV reference strains. Although these tests were applied to virus isolates, it may be possible to improve their sensitivity for direct detection in clinical material by further optimization of reaction conditions or by using the PV-specific primers as nested primers in a two-stage amplification with enterovirus group-reactive primers in the initial amplification.

An alternative approach to poliovirus-specific detection may lie in the use of immunocapture PCR, in which PV strains are selectively recovered from test specimens by attachment to paramagnetic particles or other solid phase by means of PV-specific antibody or possibly PV receptor protein. This method has been used for detection of hepatitis A virus (100, 137, 187, 291, 347) and group-specific detection of group A rotaviruses (141) in fecal and environmental samples. Achieving PV specificity during sample processing in this way would allow the use of enterovirus group-reactive primers for the initial screening assay. Intratypic differentiation would then be performed by one of the above methods.

Molecular Epidemiology

The ability to identify small differences in nucleotide sequence between virus isolates provides a sensitive tool for molecular epidemiological studies, especially of RNA viruses with a high rate of sequence evolution in nature (99, 165, 338, 375). Conventional virological techniques allow the characterization of enterovirus serotypes and intratypic differentiation of PV but do not provide sufficient information to study molecular epidemiological or evolutionary relationships between isolates or strains or to identify the source of a virus. Phylogenetic analysis of virus isolates provides the necessary information for this type of study. Genetic differences between strains may be demonstrated by oligonucleotide mapping of viral RNA (207, 282, 285), by PCR-RFLP (24, 453), or, more commonly nowadays, by nucleotide sequence determination of PCR products (307, 308, 365), which provides the most detailed and informative discrimination between strains. This method typically uses primers that target sequences which are relatively conserved among isolates of a given serotype, thus allowing the amplification of most isolates, but which flank a more variable region in the VP1-2A junction region of the genome, thus providing maximal discrimination potential between unrelated strains. The concept of PV genotype has been used to describe isolates of a single serotype which show at least 85% sequence identity in this genomic region and are therefore considered to be closely related (365).

Molecular epidemiological investigation has been used within the PEI (reviewed in reference 206) to (i) determine the sources of imported viruses (247, 307, 308, 343), (ii) monitor the pathways of virus transmission (15, 308, 365, 491), (iii) monitor the progress of control activities (423, 444), (iv) identify reservoirs sustaining virus transmission (176, 307, 365), (v) develop molecular reagents for the rapid detection of wild PV in clinical and environmental samples (94, 307, 308, 477), and (vi) provide critical evidence that PV eradication has been achieved (212, 329, 472).

Phylogenetic analysis has also been used to study the epidemiology of other enterovirus serotypes. By using this approach, it has been possible to estimate the date of emergence of newly arising enterovirus serotypes or strains (284, 285, 422) and to study their subsequent molecular evolution and global spread (50, 183, 184, 207, 243, 244), to confirm the common source of isolates from a single enterovirus outbreak (103, 105, 374) and study their genetic relationship to strains from previous outbreaks (154, 184, 227), and to identify cocirculating strains of a single serotype (172). Most of these studies have involved nucleotide sequence analysis of PCR products. However, for identification and monitoring of enterovirus outbreaks, PCR-RFLP or PCR-SSCP could allow the rapid identification of all PCR-positive samples with an identical pattern (the "outbreak pattern"), thus yielding valuable epidemiological information and reducing the requirement for complete nucleotide sequence analysis.

Resolution of Enterovirus Mixtures

The need to identify and resolve mixed enterovirus infections is evident when investigating the etiology of paralytic poliomyelitis. Failure to identify a dual PV-NPEV infection or failure to detect a wild PV or NPEV infection in a Sabin vaccine recipient may lead to misattribution of the poliomyelitis etiology, resulting in the failure to notice wild PV circulation and the delay of preventive measures. The ability to identify wild PV in sewage and other environmental samples containing a high background of NPEV or Sabin vaccine viruses is also crucial to epidemiological monitoring. The presence of multiple enterovirus types may be suspected when serotype determination by neutralization with intersecting pools or type-specific antisera is unsuccessful. Samples containing mixed Sabin and wild PV may be identified during intratypic differentiation procedures, as described above. However, recently conducted quality assurance procedures have found that failure to correctly identify enterovirus mixtures is widespread among diagnostic and reference laboratories (449). It is vital for certification of PV elimination that all PV strains are correctly identified, even when they occur in mixtures of enteroviruses.

The use of PCR-based diagnostic techniques may provide an opportunity to improve this detection rate. Methods for selective amplification of PV may be appropriate for epidemiological monitoring, where information on the possible presence of other enteroviruses is not required. However, methods that

allow the specific, simultaneous identification of individual components of enterovirus mixtures may be necessary for etiologic investigation of poliomyelitis cases or other clinical syndromes. This could be achieved by performing amplification with broadly reactive or multiplex primers, followed by molecular differentiation of each component. Some molecular differentiation methods may not detect the presence of multiple viruses. Direct nucleotide sequence analysis of PCR products produces a consensus nucleotide sequence and cannot detect minor components that comprise less than 25% of the original virus mixture (276), while PCR-RFLP analysis may give an uninterpretable result. Other methods may be more useful: multiple HCV infections have been detected with type-specific reverse hybridization probes (417, 418) or nested primers (120). Subtractive hybridization or amplification methods such as representational difference analysis (249), in which a predominant species amplified by PCR is used to enrich for minor components present in the original specimen, may also be useful if the complexity of current methods can be reduced. In one sense, all enterovirus samples are mixtures, because, like other RNA viruses, they exist as a quasispecies population. Molecular typing methods must be sufficiently robust to allow unequivocal identification of the major component(s). Because typing methods such as those discussed here based on sequence analysis or probe recognition detect consensus sequences, the presence of quasispecies does not appear to interfere.

Identification of Virulence Determinants

The wide range and severity of disease syndromes associated with enterovirus infection in humans and experimental animals are explained in part by the influence of numerous host-related factors, including age (146, 209, 252), sex (37, 253), nutritional factors (28–31, 251, 466, 467), immunodeficiency (264, 474), genetic constitution (70), preexisting immunity (223) or maternal antibody (86), and exercise (124, 166). However, the association of certain enterovirus serotypes and strains with particular disease syndromes indicates that virus-encoded factors also have a significant influence on disease manifestation. Identifying such virus-encoded factors is a worthwhile but challenging exercise. Most studies to date have been conducted with experimentally infected laboratory animals.

Cell culture and animal model studies. The search for determinants of virulence has been conducted on another level by studying enterovirus variants that show altered pathogenicity in experimental animals compared with that of the parental virus from which they were derived. Thus, the PV Sabin vaccine strains have been compared with their neurovirulent progenitors and revertants in primates and, more recently, in transgenic mice expressing the human PV receptor. By comparing nucleotide and amino acid sequences and studying the in vitro replication kinetics and in vivo virulence phenotype of chimeric viral genomes constructed from homologous neurovirulent and vaccine strain genomes, mutations that affect neurovirulence (8, 48, 55, 71, 204, 234, 254, 255, 299, 325, 337, 360, 425, 462) and temperature sensitivity (48, 71, 325, 424) have been mapped, in some cases to single nucleotides (255; reviewed in reference 278). By using a similar experimental rationale, loss of cardiovirulence in a murine model upon passage of a cardiovirulent CBV 3 strain was mapped to a single nucleotide transition at nucleotide position 234 in the 5′ UTR (438), although in a separate study of a different noncardiovirulent CBV 3 strain, no attenuating lesion could be identified in the 5′ UTR (489) or VP1-encoding region (488). In a third study with clinical CBV 3 isolates as well as laboratory-passaged strains, sequence analysis of a 300-base region of the 5′ UTR indicated that murine cardiovirulence was associated with an A residue at nucleotide position 565 whereas nonvirulent isolates had a U or C residue at this position (125). Another study involving a nonvirulent neutralization escape mutant of a cardiovirulent CBV 3 strain identified an amino acid change in the VP2-encoding region which resulted in attenuation of cardiovirulence, despite having no effect upon virus replication in the heart (222). The nonvirulent variant also failed to induce the production of tumor necrosis factor alpha upon infection of monocytes in vitro. In a murine model of CBV 4-induced pancreatitis, loss of pancreatic, hepatic, and cardiac virulence was mapped to a nucleotide change resulting in a single amino acid change in VP1 (52), with an additional minor determinant of pancreatic virulence being identified in VP4 (355).

From these studies in cell culture and animal models, it has been postulated that enterovirus virulence is determined by a number of factors, including virion thermostability, encapsidation efficiency, the specificity of cell receptors present on the virion surface which restricts the range of cell types that can be infected, and the efficiency of viral genome transcription or translation within a given cell type. Mutations affecting these functions may result in a decreased virus load, either at the site of initial virus replication or in the target organ, and may offer the immune system an advantage in restricting initial virus replication, limiting viremic spread, and eliminating virus and virus-infected cells from target organs (8, 117, 125, 234, 438).

Determinants of enterovirus virulence in humans. Investigations in which inbred or transgenic animals of uniform age, sex, and physiological status are infected with plaque-purified or cloned enteroviruses may be of value in identifying regions of the viral genome which influence disease expression. However, attenuating mutations resulting from cell culture passage of virus may not always be found in naturally occurring field strains (435). It is also probable that virulence determinants are more heterogeneous and less easily identifiable among the continually evolving enterovirus strains that cause natural infections in the genetically and physiologically diverse human population. At present, there is little information in this field, and the difficulty in identifying virulence determinants in humans should not be underestimated.

The occurrence of enterovirus epidemics may provide an opportunity to study and compare enterovirus strains which are relatively homogeneous in genetic constitution and disease association. Only a few such studies have been reported. In a comparison of two strains of ENV 71, one associated with outbreaks of hand, foot, and mouth disease and the other associated with outbreaks of aseptic meningitis or paralytic disease, significant variation of the 5′ UTR sequence was observed (492) even though the two strains were serologically indistinguishable. This variation suggested that the observed difference in neurovirulence between these strains was due to genetic differences, possibly in the 5′ UTR.

Another study in which partial 5′ UTR sequences of ECV and PV were compared found that part of an oligopyrimidine tract known to be involved in efficient binding to rRNA in neurovirulent PV strains was completely conserved in neurovirulent ECV serotypes but was variable in less neurovirulent ECV serotypes (372). It was hypothesized that this may provide a basis for explaining the differences in the observed neurovirulent potential of different ECV.

Need for further study. The above hypotheses must be tested further by studying larger numbers of field enterovirus strains isolated from patients with well-defined clinical syndromes. This could be accomplished with an appropriately designed

molecular typing system. On the basis of current knowledge of molecular determinants of virulence and tropism, a typing system targeting such candidate genomic regions as the 5′ UTR and the capsid-encoding region may, after comparison of a sufficiently large number of enteroviruses from different patient groups, permit the identification of consensus sequences associated with a particular disease phenotype. The biological effect of such consensus sequences could then be studied in cell culture and, where possible, in animal models with chimeric viruses generated by intertypic genome recombination or site-directed mutagenesis. Although this is a formidable task, the information provided by such investigations would further increase our understanding of the pathogenesis of enterovirus infection. This information may provide a rationale for the design of recombinant live-attenuated enterovirus vaccines (62) or antiviral prophylactic or therapeutic measures.

Classification of Enteroviruses Based on Receptor Usage

Viral infection of host cells is initiated by binding of the virus to a specific cell surface protein. The requirement of a virus for a specific cell surface protein and the cellular and tissue distribution of this protein clearly may influence viral tropism and disease manifestation (162). It is presumed that enteroviruses have specific regions in their symmetrical capsid structure, called viral attachment proteins (VAP), which recognize specific features of selected cellular membrane proteins as receptors. Such specific receptors are an important determinant of the host range and cell and tissue tropism of a virus. These receptors are considered to possess two functions: first, to attach virions, and second, to eclipse virus infectivity. The identification and characterization of enterovirus receptors has been the object of intensive investigation in recent years, because knowledge of virus-receptor interaction as the initial step of infection offers an opportunity for chemical or immunologic intervention before the viral genome has reached relative stability within the cell. The two most obvious possibilities for antiviral therapy based on knowledge of virus receptors involve the blocking of virus-receptor interactions with either antireceptor antibodies or the soluble receptor and the development of pharmacological agents that specifically block the virus binding at the level of either the cell receptor or the VAP. However, cell receptors have been identified for only a small number of enterovirus types thus far.

For PV, the receptor is known to be a member of the Ig superfamily (274). The integrin VLA-2 has been reported to be the receptor for ECV 1 (36), and decay-accelerating factor (DAF/CD55), a membrane protein of 70 kDa, has been reported to be the receptor for ECV 3, 6, 7, 11, 12, 20, and 21 (34) and ENV 70 (203). Like the major group of human rhinoviruses, CAV 13, CAV 15, CAV 18, and CAV 21 can use intercellular cell adhesion molecule 1 as the receptor (80) whereas CAV 9 interacts with the vitronectin receptor (371). From infected HeLa cells, a CBV-receptor complex containing virus capsid proteins bound to a cellular protein of 46 kDa was purified (35, 257). Shafren et al. (399) reported that DAF/CD55 might also be a cell attachment receptor for serotypes B1, B3, and B5. However, competition experiments revealed that all six serotypes of CBV compete for a common receptor protein. A 100-kDa cellular protein that also has the ability to bind CBV 1 to CBV 6 was recently identified by Raab de Verdugo et al. (352).

Some information is available on the regions of the enterovirus capsid involved in cell attachment. PV strains are hypothesized to attach to target cells in a manner similar to human rhinoviruses via a "canyon" which is defined by multiple viral capsid peptides (373). A similar surface structure thought to serve as the primary and secondary receptor-binding site has also been described for CBV 3 (300).

It appears that the attachment of CAV 9, ECV 22, and ECV 23 is mediated by a portion of VP1 which contains a highly conserved 3-amino-acid sequence, the RGD (Arg-Gly-Asp) motif (60, 415). In CAV 9, this RGD motif is located within a relative 15-amino-acid insert in comparison to other enteroviruses. The RGD motif is known to play a role in cell adhesion in many proteins present in extracellular matrices and in the blood. In all three RGD-containing enteroviruses as well as in foot-and-mouth disease virus (259), blocking experiments with specific peptides showed the significance of the RGD motif for the infection of cells. However, CAV 9 can also infect some cells independently of the RGD motif (370). The identification of capsid regions of other enteroviruses involved in receptor binding would provide the basis for a molecular classification of enteroviruses according to cell receptor usage. In the future, this may lead to a greater understanding of the influence of receptor specificity on viral tropism and pathogenicity and may allow the targeting of antiviral therapy with agents designed to interfere with specific virus-receptor interactions.

Determination of Sensitivity or Resistance to Antiviral Agents

A range of antipicornavirus agents have been described in the literature (reviewed in references 12 and 319). However, to date, none appear likely to play a potential role in clinical practice. Most act by noncovalent interaction with virus capsids, resulting in inhibition of viral attachment to cell receptors or inhibition of disassembly following cell entry. Kraus et al. (232) isolated a rhodanine-resistant variant of ECV 12 by sequential passage in the presence of rhodanine, an inhibitor of virion disassembly. Sequence analysis of the resistant variant and parental virus revealed seven amino acid differences, six of which were located in the capsid-encoding region. The amino acid differences responsible for the rhodanine-resistant phenotype have not yet been identified. Similarly, resistance to enviroxime, an inhibitor of picornavirus RNA replication, has been mapped to the 3A protein, and amino acid substitutions conferring resistance have been mapped for human rhinovirus 14, PV 1 (158), and CBV 3 (159). If clinically useful antiviral agents were identified, it would be necessary to monitor drug resistance. Since resistance might result from numerous different mutations, it may not prove possible to devise a molecular typing system which identifies every possible mutation. Experience with drug resistance in HIV and cytomegalovirus infections has found that most cases of resistance are caused by one or more of a few common mutations, which can be readily identified by nucleotide sequencing of PCR products (188, 194, 364), differential hybridization of amplified viral RNA to oligonucleotide probes (129), or point mutation assays (44, 118, 205). However, these tests may fail to predict resistance resulting from other less common mutations (402). Prediction of drug susceptibility based on nucleotide sequence data will become increasingly difficult as new combination and sequential chemotherapeutic strategies are introduced.

Formation of a Database of Enterovirus Genome Sequences

Molecular sciences are heavily dependent on the use of computers for accumulation, processing, analysis, storage, and dissemination of experimental data, and the role of transnational nucleic acid and protein sequence databases in these

processes is crucial (reviewed in reference 275). Due to the increasing use of general sequence databases and the requirement for specialist functions, a number of specialist databases which cross-reference with major sequence databases have been formed (195). Even if an enterovirus molecular typing system were to use methods other than complete nucleotide sequencing of PCR products, it is likely that nucleotide sequencing of representative samples would still be required for ongoing quality assurance, discrepancy analysis, and resolution of untypeable samples. A database of enterovirus sequences generated by molecular detection and typing methods would facilitate the quality assurance of detection and typing reagents and provide a resource for the study of viral epidemiology, evolution, taxonomy, and molecular pathogenesis.

MOLECULAR TYPING OF ENTEROVIRUSES: THE WAY FORWARD

The development of a universally applicable molecular typing system for enteroviruses will require considerable effort to generate additional molecular sequence data, to provide reference reagents, to research laboratory and computer methods required for the development of a typing system, and to raise the funding required to make this work possible.

Identification of Priorities for Molecular Typing

Molecular typing methods for viruses should aim to provide clinically and biologically useful information about field viruses, particularly with regard to virulence, viral epidemiology, and virus serotype identification. The extent to which molecular classification systems for viruses have aimed toward or achieved these goals varies, and in some cases further development and refinement of the methods are required. For enteroviruses, to these general aims must be added the requirement to differentiate wild PV and NPEV, at least until global eradication of wild PV is verified.

A genotyping method based on phylogenetic relationships would not achieve all these objectives but would be of value for epidemiological studies and may be useful as an initial step in further typing procedures. Phylogenetic typing is also a realistic intermediate target for enterovirus molecular typing, and methods such as those described by Arola et al. (19), based on nucleotide sequence analysis of a target region located in the 5′ UTR and the VP4- and VP2-encoding region, should be pursued further.

Even if wild PV eradication is achieved by the year 2000, a period of continued surveillance will be required to verify this. The ability to differentiate between wild and vaccine-derived PV and NPEV is therefore likely to be required until at least 2010. In view of this, molecular detection of wild PV should also be considered a goal of research and development of molecular typing, which should be achievable by methods discussed above.

In this review, we have considered a number of biological characteristics of enteroviruses for which typing methods may be useful. In most cases, our understanding of these characteristics is incomplete. The development of molecular typing methods based on these biological phenotypes will therefore require further preliminary research to identify the relevant molecular determinants and will doubtless prove more refractory to genetic analysis, but it should nonetheless be considered a longer-term aim. Without such objectives, it is doubtful whether a molecular typing system would become widely used.

Establishment of a Collection of Representative Enterovirus Strains

It will be apparent that the development of molecular typing will require access to prototype enterovirus strains; field enterovirus isolates, including well-characterized isolates, prime strains, and untypeable isolates collected during recent years and from different geographical regions; and clinical specimens in which enteroviruses have been detected by culture or by PCR. Relevant demographic and clinical information about the source of clinical isolates and specimens must also be recorded to allow statistical correlation between genotypic and phenotypic features of viruses. Such a collection would provide a resource for the generation of molecular sequence data and for the evaluation of subsequently developed molecular typing assays.

Augmentation of Enterovirus Molecular Sequence Data

Although the volume of molecular sequence data available for human enterovirus genomes has increased in recent years, much additional sequence data is still required for many prototype strains, particularly for ECV and CAV. Another important area where information is needed is the degree of sequence variation within individual serotypes. Limited information on this is available only for a few serotypes (Table 3). A third relevant area is the study of sequences obtained directly by PCR amplification from patient specimens and their comparison with sequences of the corresponding cell culture isolates, since selective susceptibility of cultured cells and the process of adaptation to growth in cell culture may result in selection of a virus population which is substantially different in genetic constitution and biological phenotype from those of the original field strain (72, 74, 157).

The genomic regions that should be targeted for sequence analysis will be determined by the objectives of molecular typing. Phylogenetic classification may be based on the sequence of the VP2-encoding region or the length of the 3′ UTR, as described above. Classification based on biological features such as serotype, receptor usage, tissue tropism, or virulence will require sequence information from the genomic regions that influence these properties. VP1 and VP2 proteins contain the major neutralizing epitopes, and also the VAP involved in cell receptor interaction, and this genomic region should also be investigated as a target for molecular typing methods. Observations that mutations in VP3 and VP4 may also affect virion surface features and biological properties suggest that this genomic region should also be analyzed. The 5′ UTR is also of special interest in view of its role in the regulation of transcription and translation of the viral genome and its influence on virulence, and this region should be considered an important target for comparative sequence analysis. In contrast, the nonstructural protein-encoding region is relatively conserved in sequence and possibly also in function among enteroviruses and is not generally believed to contribute significantly to the diversity of enterovirus biology. It is therefore likely that a detailed knowledge of the structure and function of the 5′ half of the enterovirus genome would provide a sufficient basis for understanding the observed biological variation of enteroviruses and for designing molecular typing systems to classify and identify enteroviruses according to this variation.

The most straightforward way to obtain additional sequence information is probably by direct sequencing of PCR products generated with consensus, degenerate, or multiplex primers whenever possible (177, 349). This method could be applied to prototype strains and clinical isolates as well as to PCR-posi-

tive patient specimens. For example, amplification of the complete 5′ UTR may readily be achieved; sequencing of PCR products could then be performed with both external PCR primers and internal forward and reverse primers. The two inner primers could be located in the region around base 450, which is highly conserved throughout the enterovirus genus. Multiplex or degenerate PCR primers would probably be required to allow the detection of the two major 5′ UTR phylogenetic subgroups (Fig. 2), as suggested by Pöyry et al. (342).

These molecular sequence data would provide a basis for the development of molecular typing methods involving current or future nucleic acid or protein technologies.

Design and Development of a Molecular Typing System

Development of a typing system will require a strong research base to determine the molecular basis of distinguishing the biological characteristics of enteroviruses. Evaluation will be required to determine the reliability of identifying specific genomic sequences as specific markers of virus type. This is likely to prove most difficult when biological features are associated with higher-order structures dependent on the interaction of noncontiguous nucleotide or amino acid sequences. Refinement of target sequences and optimization of reagents and methods over time will undoubtedly be required. Ongoing evaluation and quality assurance will also be necessary to ensure that typing methods retain specificity and sensitivity in identifying current enterovirus strains.

It is envisaged that molecular typing of enteroviruses would be conducted primarily in regional or national reference centers, as is often the case with current serotyping methods. Specialist procedures, such as intratypic differentiation of PV, would probably still be performed in WHO reference laboratories. While methods for epidemiological investigation of specific outbreaks may be tailored to local or temporary requirements, a universally applicable molecular typing system is desirable for most typing purposes. A molecular typing system for enteroviruses should therefore be simpler to perform and interpret than serotyping by neutralization and should be more amenable to standardization and quality control. Reagents should be temperature stable and the test should be robust enough to be performed in diverse climates. Results should be rapidly available, to maximize clinical utility. Molecular typing methods should be adaptable, to accommodate emerging and evolving virus strains, and should be cost-effective.

To realize these goals, research and development should make full use of recent advances in PCR technology and of automation of sample preparation, PCR, and post-PCR processing, including nucleotide sequencing. The choice of post-PCR analysis in particular will be influenced by these constraints. Electrophoretic analysis is difficult to standardize and automate. PCR-RFLP, PCR-SSCP, and other electrophoretic sizing procedures should therefore be avoided. Methods that require multiple parallel tests to identify different virus types will be both labor- and reagent-intensive and unlikely to be cost-effective or to give rapid results. A rapid and definitive typing result is most likely to be achieved by reverse hybridization of the PCR product to solid-phase-bound, type-specific probes in a microtiter plate-based, microchip, or line probe assay or by direct nucleotide sequence analysis of PCR products. The latter has been made more feasible by the recent introduction of rapid, cheaper automated methods. Sequence analysis may be required for epidemiological studies and quality assurance, whereas probe hybridization analysis may be sufficient and more practical for other purposes and will probably be necessary for identification of mixed infections. However, the ongoing development of DNA and protein technology may provide other possible methods in the near future.

Establishment of a Network of Laboratories

It will be apparent that the work required to develop a reliable and useful molecular typing system for enteroviruses is extensive and will require considerable long-term funding. A strategy to raise this funding must address the aforementioned perception of enteroviruses as relatively unimportant agents of human disease and the current reality of decreased funding in most areas of basic research. At present, enterovirus research is conducted mainly in laboratories with independent funding studying such aspects as pathogenesis, structure and molecular biology, epidemiology, diagnosis, and biodiversity. All these areas of interest have relevance to the design of molecular typing methods. A collaborative network of such laboratories would build on the expertise of individual research groups and provide the critical mass required to sustain central facilities required for archiving reference reagents and sequence data and coordination of research efforts. It is also to be hoped that such a grouping would achieve greater prominence in areas of public education and so convince the medical and scientific communities, as well as the general public, of the need to continue research into this important genus of viruses and the potential benefits of new ways of typing human enteroviruses.

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APPENDIX

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