

***In situ* detection of enteroviral genomes in myocardial cells by nucleic acid hybridization: An approach to the diagnosis of viral heart disease**

(coxsackievirus B3 cDNA/myocarditis/dilated cardiomyopathy/endomyocardial biopsy/mouse model)

REINHARD KANDOLF*[†], DETLEV AMEIS*, PHILIP KIRSCHNER*, ANNIE CANU[‡],
AND PETER HANS HOFSCHEIDER*

*Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany; [†]Medizinische Klinik I, Klinikum Grosshadern, Universität München, D-8000 München 70, Federal Republic of Germany; [‡]Département de Microbiologie, Unité de Formation et de Recherche des Sciences Pharmaceutiques, F-14000 Caen, France

Communicated by G. Melchers, May 27, 1987 (received for review April 13, 1987)

ABSTRACT We have developed an *in situ* hybridization assay capable of detecting enteroviral RNA in myocardial cells, using molecularly cloned coxsackievirus B3 cDNA as a diagnostic probe. Because of the high degree of nucleic acid sequence homology among the numerous enteroviral serotypes, including the group A and B coxsackieviruses and the echoviruses, detection of these various agents commonly implicated in human viral heart disease is possible in a single hybridization assay. We demonstrate the considerable potential of this method for an unequivocal diagnosis of enteroviral heart disease as well as for pathogenicity studies. Using athymic mice persistently infected with coxsackievirus B3 as a model system, we show that the myocardium is affected in a disseminated, multifocal manner.

In North America and Europe, acute myocarditis is most commonly associated with infections by coxsackie B viruses (types 1 to 5). Other members of the human enteroviruses comprising at present over 70 serotypes (e.g., various coxsackie A viruses and echoviruses) are also considered to be relatively frequent causes of human viral heart disease (1-4). These agents appear to be capable of producing dilated cardiomyopathy of acute onset or lead to a variety of cardiac arrhythmias. Some of the acute cases may also evolve into a chronic form of dilated cardiomyopathy.

The difficulty of establishing a specific diagnosis of viral heart disease is a major problem in clinical cardiology. Confirmation of the clinical suspicion of viral heart disease demands demonstration of replicating virus inside myocardial cells, which is exceedingly difficult by conventional methods (5). In addition, the increasing availability of potential antiviral agents has accentuated the need for methods by which endomyocardial biopsy specimens of patients with suspected viral heart disease can be diagnosed conclusively (6, 7).

In order to introduce *in situ* nucleic acid hybridization (8-10) as a diagnostic tool in suspected enteroviral heart disease, we have recently cloned the single-stranded RNA genome of coxsackievirus B3 (CVB3) (11) that had been propagated in cultured human heart cells (12). Full-length reverse-transcribed cloned viral cDNA generated infectious CVB3 upon transfection into mammalian cells, demonstrating the molecular cloning of a faithful transcript of the original viral RNA. We now report the use of radioactively labeled cloned CVB3 cDNA as a diagnostic probe for the *in situ* detection of viral RNA in infected cultured cells and in myocardial tissue sections of CVB3-infected T-cell-deficient mice. We show that the molecular hybridization approach

permits the detection of infected myocardial cells at the single-cell level, thus providing a unique possibility for an unequivocal diagnosis.

A further advantage of the nucleic acid hybridization approach is provided by the high degree of genetic homology shared among the different serotypes of the human enterovirus group (11, 13-17), making possible their detection in a single hybridization assay. Detection of various enteroviruses by use of subgenomic cDNA fragments has been reported (18-20). Here we demonstrate that the complete cloned CVB3 cDNA can be used as an enteroviral diagnostic reagent for the detection of the main etiologic agents of human viral heart disease.

MATERIALS AND METHODS

Viruses, Cells, and Animals. Coxsackie B viruses (types 1 to 6) were obtained from the American Type Culture Collection (ATCC VR-28, VR-29, VR-30, VR-184, VR-185, and VR-155) and plaque-purified three times on Vero cells (ATCC CCL 81) as described (12). Plaque-purified coxsackie B viruses were further passaged twice in Vero cells for the preparation of seed virus. In addition, coxsackievirus A9 (CVA9), echoviruses 11 and 12, poliovirus type 1, and vesicular stomatitis virus were propagated in Vero cells.

Athymic mice (NMRI *nu/nu*), 5 weeks old, were inoculated intraperitoneally with 50 μ l of Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline containing 5×10^5 plaque-forming units (pfu) of CVB3. All these mice developed myocardial lesions in the presence of infectious CVB3 as observed up to 56 days after infection. Aseptically removed hearts were quick-frozen in liquid N_2 and stored at -80°C .

Preparation of Cells and Tissue Sections. Microscope slides were pretreated by incubation for 3 hr at 65°C in $3 \times \text{SSC}$ (0.45 M NaCl/0.045 M trisodium citrate) containing 0.02% (wt/vol) each bovine serum albumin, polyvinylpyrrolidone, and Ficoll (21), dipped briefly in distilled water, and fixed for 20 min at room temperature in ethanol/acetic acid (3:1, vol/vol).

Confluent monolayers of Vero cells were infected at different multiplicities of infection (moi) with CVB3 (Nancy strain) and harvested by trypsinization 1 and 5 hr after infection. Cells were washed twice in Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline and deposited on pretreated slides. After air-drying, cells were fixed for 20 min at room temperature in ethanol/acetic acid (3:1, vol/vol) and stored at -80°C in ethanol until used for hybridization.

Ten-micrometer-thick frozen sections of CVB3-infected mouse myocardial tissue were prepared at -20°C , deposited

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CVB3, coxsackievirus B3; CVA9, coxsackievirus A9; moi, multiplicity of infection; pfu, plaque-forming units.

on pretreated slides, air-dried thoroughly, and fixed as described above for Vero cells.

Preparation of Labeled DNA Probes. The infectious CVB3 cDNA clone pCB3-M1 (11) was digested with *Kpn* I or *Bam*HI restriction endonuclease to generate a 6.2-kilobase and a 1.0-kilobase fragment, respectively. These two CVB3-specific fragments represent $\approx 95\%$ of the viral genome excluding 66 nucleotides at the 5' end and ≈ 300 nucleotides of the polyadenylated 3' end. After the two CVB3 cDNA fragments were purified twice by agarose gel electrophoresis, they were used in different assays for nick-translation (22) under conditions to generate radiolabeled CVB3 cDNA fragments ≈ 100 nucleotides long. CVB3 cDNA fragments (200–500 ng) were incubated for 2 hr at 15°C in 20 μ l of 50 mM Tris-HCl, pH 7.5/5 mM MgCl₂/bovine serum albumin (5 μ g/ml)/10 mM 2-mercaptoethanol/20 μ M each of the four [³H]dNTPs (20–110 Ci/mmol; 1 Ci = 37 GBq)/DNase I (0.01–0.1 μ g/ml)/DNA polymerase I (250 units/ml). The nick-translated CVB3 cDNA fragments were isolated by phenol extraction followed by spin-column chromatography (centrifugation through a Sephadex G-50 gel-filtration column) and mixed at a molar ratio of 1:1. The specific activity of the ³H-labeled CVB3 cDNA probe was 0.5–1 $\times 10^8$ dpm/ μ g of DNA. In addition, probes were prepared with [α -³⁵S]thio]dATP and [α -³⁵S]thio]dCTP (1200 Ci/mmol) to a specific activity of 10⁸ dpm/ μ g of DNA. Control DNA probes were prepared from the plasmid vector p2732B (11).

In Situ Hybridization. Before hybridization the slide preparations were treated as follows: 20 min at room temperature in 0.2 M HCl, 30 min at 70°C in 2 \times SSC, and 15 min at 37°C in 20 mM Tris-HCl, pH 7.4/2 mM CaCl₂/proteinase K (1 μ g/ml) (10). Slides were washed twice in distilled water and then dehydrated in graded ethanol solutions (70% and 95%). Hybridization mixture contained ³H- or ³⁵S-labeled CVB3 cDNA probe (200 ng/ml, heated to 100°C for 5 min), 10 mM Tris-HCl (pH 7.4), 50% (vol/vol) deionized formamide, 600 mM NaCl, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.05% bovine serum albumin, 10% (wt/vol) dextran sulfate, 10 mM dithiothreitol, sonicated denatured calf thymus DNA (200 μ g/ml), myocardial total RNA (1 mg/ml), and rabbit liver tRNA (200 μ g/ml). Fourteen microliters of hybridization mixture was applied to each slide, siliconized coverslips were mounted and sealed with rubber cement, and hybridization was allowed to proceed at 25°C for 48 hr. Slides were then washed for 18 hr at 37°C in 10 mM Tris-HCl, pH 7.4/2 \times SSC/50% formamide/1 mM EDTA followed by 1 hr at 55°C in 2 \times SSC, then rinsed in 2 \times SSC, and dehydrated in graded ethanol solutions containing 300 mM ammonium

acetate. Hybridized preparations were autoradiographed with NTB2 nuclear track emulsion (Eastman) diluted 1:1 with 600 mM ammonium acetate. After exposure for indicated intervals at 4°C, slides were developed with Kodak D19 and examined unstained with a Zeiss interference contrast microscope.

RNA Blot Analysis of Enterovirus-Infected Cells. Vero cells were inoculated with different enteroviral strains (CVB1, CVB2, CVB3, CVB4, CVB5, CVB6, CVA9, echo 11, echo 12, polio type 1) at a moi of 20 pfu per cell. Total RNA of infected cells was isolated when 50% of the cells exhibited cytopathologic effects, applied in 1:10 dilution (1 μ g, 0.1 μ g) to nitrocellulose paper by means of a slot-blot filter apparatus, and hybridized with ³²P-labeled CVB3 cDNA corresponding to the region from 0.06 to 7.2 kilobases of the viral genome (11). CVB3 RNA isolated from suspensions of purified CVB3 was used as a positive control and loaded in the presence of total RNA from uninfected Vero cells. Purified RNA from foot-and-mouth-disease virus and total RNA from cells infected with vesicular stomatitis virus served as negative controls.

Materials. Sources were as follows: *Escherichia coli* DNA polymerase I, Pharmacia; bovine pancreatic DNase I and restriction enzymes, Boehringer Mannheim; proteinase K, Merck (Darmstadt, F.R.G.); [³H]dNTPs (20–110 Ci/mmol), [α -³⁵S]thio]dATP (1200 Ci/mmol), [α -³⁵S]thio]dCTP (1200 Ci/mmol), and [α -³²P]dCTP (3000 Ci/mmol), Amersham.

RESULTS

Specificity of In Situ Hybridization for CVB3 RNA. Specificity of the *in situ* hybridization method was shown by use of Vero cells, a permanent cell line of African green monkey kidney cells. Uninfected Vero cells exhibited essentially no autoradiographic grains when probed with the cloned and restriction endonuclease-derived ³H-labeled fragments of CVB3 cDNA (Fig. 1A). In contrast, highly significant labeling was observed when CVB3-infected Vero cells were hybridized with the CVB3 cDNA probe. Fig. 1B shows, as an example, Vero cells that were infected with CVB3 at a low moi (0.5 pfu per cell) and then hybridized 5 hr after infection. Cells replicating viral RNA are clearly distinguishable from uninfected cells. In contrast to cultures infected with a low moi of 0.5 pfu, virtually 100% of the cells were labeled when hybridized 5 hr after infection with CVB3 at a high moi (50 pfu per cell; Fig. 1C). No labeling was observed when CVB3-infected Vero cells were hybridized with the plasmid p2732B control DNA probe. Moreover, no labeling was found with preparations of infected cells that were hybridized with the

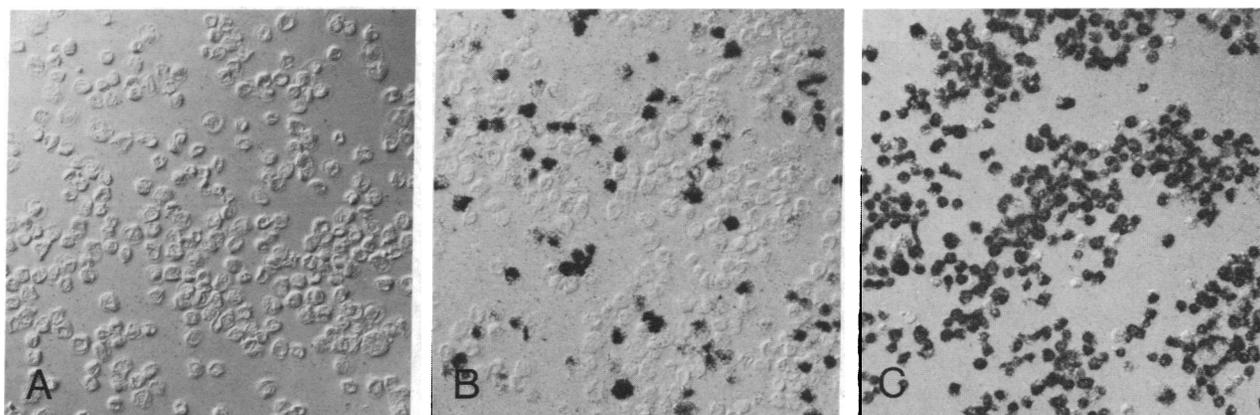


FIG. 1. Specificity of *in situ* hybridization of the restriction endonuclease-derived ³H-labeled CVB3 cDNA fragments to uninfected and infected Vero cells. Cells were deposited on pretreated slides, hybridized *in situ*, and exposed for 2 weeks. (A) Uninfected Vero cells. (B) Vero cells harvested 5 hr after infection with CVB3 at a low moi (0.5 pfu per cell). (C) Vero cells harvested 5 hr after infection with CVB3 at a high moi (50 pfu per cell). (Interference contrast; $\times 200$.)

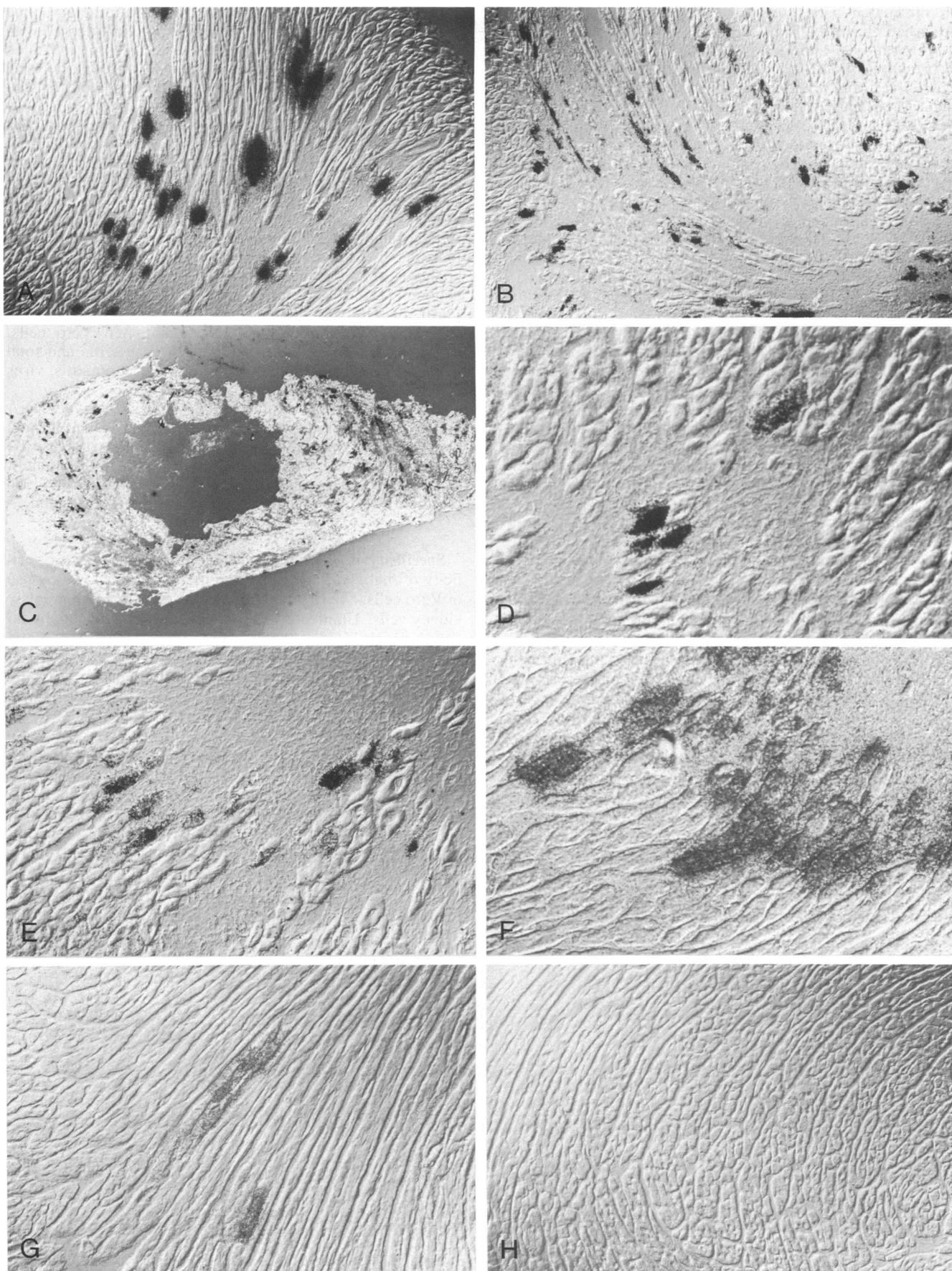


FIG. 2. (Legend appears at the bottom of the opposite page.)

CVB3 cDNA probe after treatment with RNase A at 100 $\mu\text{g/ml}$ for 1 hr at 37°C (data not shown). Thus, the observation of grains after hybridization with ^3H -labeled CVB3 cDNA correlated conclusively with the presence of CVB3, indicating that the *in situ* hybridization was specific for the presence of viral nucleic acid.

***In Situ* Detection of CVB3 RNA in Infected Mouse Myocardial Tissue.** To determine optimal processing conditions for *in situ* detection of enteroviral RNA in heart muscle, we examined 100 myocardial tissue sections of infected mice together with 50 control sections of uninfected mice. CVB3 was administered intraperitoneally to adult athymic mice and the presence of viral RNA was determined in 10- μm sections of the myocardium up to 56 days after infection (Fig. 2). The autoradiographic silver grains, which indicate hybridization between viral RNA and radiolabeled cloned CVB3 cDNA, are clearly related to distinct infected myocytes (Fig. 2A). These cells are easily identified by interference contrast microscopy in unstained sections because of their characteristic size and morphology. In this model system of enteroviral heart disease, myocardial involvement was found to be multifocal and randomly distributed in the heart muscle (Fig. 2B). Myocardial cross-sections revealed a transmural disseminated infection of the myocardium as demonstrated in Fig. 2C for the left ventricle. Infected myocytes were often found in clusters within areas of severe lesions and myocardial fibrosis (Fig. 2D). Furthermore, progression of the infection could be observed from areas with myocardial fibrosis to as yet uninfected myocytes (Fig. 2E and F), indicating the possible cell-to-cell spread of the virus. However, viral RNA was also found in isolated myocytes in apparently normal myocardial tissue, which was primarily observed in the early stage of the infection (Fig. 2G). In addition, viral RNA appeared also to be located within the small interstitial myocardial cells (Fig. 2B and E).

No labeling of myocardial cells was found when infected myocardial sections were probed with the radiolabeled plasmid p2732B control DNA or with the cloned *EcoRI* J fragment of the genetically unrelated cytomegalovirus (23) (data not shown). In addition, no labeling of myocardial cells was found when myocardial tissue sections of uninfected mice were probed with the radiolabeled CVB3 cDNA (Fig. 2H). Even after autoradiographic exposure times of up to 10 weeks, negative controls showed extremely low levels of background silver grains.

Sensitivity of *In Situ* Hybridization Method. To quantitate the number of viral RNA copies per cell, CVB3-infected Vero cells from the same culture were analyzed by *in situ* hybridization as well as by RNA blot hybridization. Cells harvested 1 hr after infection, containing 20 viral genomes per cell (as calculated by RNA blot hybridization), averaged about 25 grains per cell over background within 2 weeks of autoradiographic exposure after hybridization with the ^{35}S -labeled cDNA probe (as determined by examining 100 randomly selected cells). Cells harvested 5 hr after infection, containing 5×10^4 to 1×10^5 viral genomes per cell, showed high labeling within 2 days of exposure after hybridization with the ^{35}S -labeled probe (data not shown). Positive hybridization signals with infected myocardial tissue hybridized *in situ* under identical conditions was observed also within 2 days of exposure (Fig. 2G), indicating high copy numbers of replicating viral RNA in myocardial cells.

Cloned CVB3 cDNA as an Enteroviral Diagnostic Reagent. Recent work in our laboratory (11) and elsewhere (18–20) had

shown that nucleic acid hybridization using cDNA probes derived from the RNA of certain enteroviruses can recognize diverse enteroviral serotypes. To estimate the degree of nucleotide sequence homology between the numerous cardiotropic enteroviral serotypes by a slot-blot hybridization assay, Vero cells were infected with various enteroviruses at a moi of 20 pfu per cell. Total RNA of infected cultures was isolated when about 50% of cells exhibited cytopathologic effects, blotted on nitrocellulose filter in 1:10 dilutions, and hybridized with ^{32}P -labeled CVB3 cDNA corresponding to the region from 0.06 to 7.2 kilobases of the viral genome. The results (Fig. 3) indicate a remarkably high degree of nucleotide sequence homology among different human enteroviral strains, including coxsackie B viruses (types 1 to 6), coxsackievirus A9 (CVA9), echoviruses 11 and 12, and poliovirus type 1. Because comparative nucleotide sequence data for CVB3 (11, 15, 16) and poliovirus type 1 (13, 14) show an overall homology of about 70% within functional genes (e.g., the replicase gene) or within the noncoding 5' region, these cross-hybridization data (Fig. 3) appear to predict an even higher degree of nucleotide sequence homology between the different serotypes of coxsackie A and B viruses as well as echoviruses. With respect to group B coxsackieviruses, the extent of cross-hybridization is in agreement with the recently published sequence of CVB1 (17), which indicated a homology of 85% for regions like the replicase gene of these viruses. In contrast, foot-and-mouth-disease virus, a nonhuman picornavirus or vesicular stomatitis virus do not hybridize under the conditions described. These results indicate that the complete cloned CVB3 cDNA can be used as a human enteroviral diagnostic reagent. Using a CVB3 cDNA fragment corresponding to the region from 0.88 to 2.7 kilobases of the viral genome, which encodes the viral coat proteins 1B (VP2), 1C (VP3), and parts of 1A (VP4) and 1D (VP1) (24), we found, in agreement with others (19), that serotype-specific hybridization is possible for the CVB3 RNA (data not shown).

DISCUSSION

Enteroviruses of the human Picornaviridae, such as the group A and B coxsackieviruses and the echoviruses, are generally considered to be the most common agents of viral heart disease. However, physicians lack information on how often enteroviruses are actually the cause of dilated cardiomyopathy, what the prognosis is in such cases, and whether specific therapy can affect prognosis. The reason for this fundamental lack of knowledge is the difficulty of obtaining an unequivocal diagnosis of suspected viral heart disease.

The techniques so far used to investigate endomyocardial biopsy specimens from patients with suspected viral heart disease include electron microscopy, virus-isolation procedures, and immunohistochemistry. However, with respect to enteroviral heart disease, these techniques are limited by various methodological problems: (i) at the electron microscope level, enteroviruses are indistinguishable in density and size from ribosomes (25); (ii) virus-isolation procedures appear to be successful only in the acute phase of the disease with extensive virus replication (2, 5); (iii) immunohistochemistry using serotype-specific antibodies is less practicable, as is enterovirus serology, because of the high number of distinct enteroviral serotypes that might be implicated in the disease.

Using cloned CVB3 cDNA as a diagnostic reagent, detection of myocardial cells replicating enteroviral RNA is now

FIG. 2. Autoradiographs of CVB3-infected (A–G) and uninfected (H) mouse myocardial tissue hybridized *in situ* with ^3H -labeled (B–E) or ^{35}S -labeled (A, F–H) cloned CVB3 cDNA probe. Days after infection were 56 for A, 23 for B–E, 42 for F, and 4 for G. Exposure times were 9 days for A and H, 6 weeks for B–E, 4 days for F, and 2 days for G. Note that since silver grains are positioned at various levels within the photoemulsion, some grains are not observed and appear out of focus in photography. [Interference contrast microscopy of unstained sections; $\times 780$ (A, G, and H); $\times 90$ (B), $\times 25$ (C), $\times 360$ (D and E), $\times 585$ (F).]

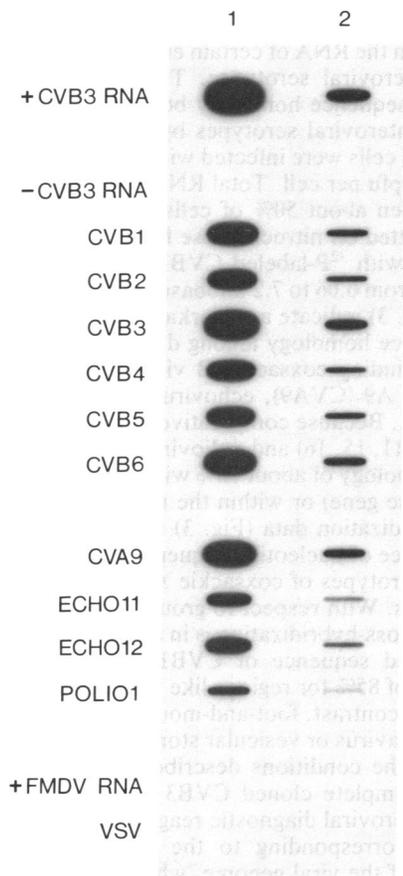


FIG. 3. Hybridization of cloned CVB3 cDNA to total RNA from Vero cells infected with various enteroviruses. Cells were infected with coxsackie B viruses (CVB1, CVB2, CVB3, CVB4, CVB5, CVB6), coxsackievirus A9 (CVA9), echovirus 11, echovirus 12, or poliovirus type 1. Total RNA was isolated when about 50% of the cells exhibited cytopathologic effects. One microgram (column 1) and 0.1 μ g (column 2) of total cellular RNA were applied to nitrocellulose paper and hybridized with 32 P-labeled cDNA fragments corresponding to the region from 0.06 to 7.2 kilobases of the viral genome. Reference slots (+CVB3 RNA) consisted of 5 ng (column 1) and 0.5 ng (column 2) of purified CVB3 RNA and were loaded in the presence of 1 μ g (column 1) and 0.1 μ g (column 2) of total uninfected Vero cell RNA, respectively. Analogously, purified RNA of foot-and-mouth-disease virus (+FMDV RNA) was applied in the presence of Vero cell RNA. Uninfected Vero cell RNA (-CVB3 RNA) served as a negative control. Vesicular stomatitis virus (VSV) RNA was obtained by isolating total RNA from infected Vero cells. Autoradiographic exposure was at -80°C for 18 hr.

possible at the single-cell level. We have developed an *in situ* hybridization approach that proved to be a powerful method not only for establishing an unequivocal diagnosis of myocardial infection but also for study of the pathology of the disease. Analysis of the mouse model of dilated-type cardiomyopathy revealed a multifocal transmural involvement of the heart muscle with progression of the infection from areas with myocardial fibrosis to uninfected cells (Fig. 2).

High sensitivity of detection was made possible by several improvements in methodology, including optimized hybridization conditions to prevent nonspecific binding and use of radiolabeled cloned cDNA fragments of about 100 nucleotides in length. Quantification of CVB3 copy numbers in infected Vero cells by RNA blot analysis and comparison with *in situ* hybridization of cells from the same culture indicated that as few as 20 viral copies per cell are detectable within 2 weeks of autoradiographic exposure. Furthermore,

overexposed myocardial slide preparations showed extremely low background signals, demonstrating the high specificity of *in situ* hybridization for the detection of enteroviral RNA.

As shown by cross-hybridization experiments, detection of the most commonly implicated agents of human viral heart disease, including group A and B coxsackieviruses as well as echoviruses, is possible in a single hybridization assay. This broad detection spectrum will greatly facilitate diagnosis of suspected enteroviral heart disease, since from the clinical point of view the antigenic typing of an etiologically implicated enteroviral strain appears to be of secondary importance and can be carried out later—e.g., by standard virological techniques or by hybridization with serotype-specific cDNA fragments.

Positive hybridization results obtained with human endomyocardial biopsy samples of patients with acute or chronic myocarditis as well as dilated cardiomyopathy indicate the general applicability of the method described for the diagnosis of enteroviral heart disease (7). It should now be possible with this technique to address a number of important questions concerning the molecular basis of pathogenicity. We are especially interested in the question of enteroviral persistence in the pathogenesis of chronic dilated cardiomyopathy evolving from acute or subacute myocardial infection.

Echoviruses 11 and 12, CVA9, and poliovirus type 1 were kindly provided by Dr. H. J. Eggers (University of Cologne, Cologne, F.R.G.). Foot-and-mouth-disease virus O₁K RNA was a generous gift from Dr. K. Strohmaier (Federal Research Institute for Animal Virus Diseases, Tübingen, F.R.G.). This work was supported in part by Grants Ka593/2-1 and Am65/1-1 from the Deutsche Forschungsgemeinschaft and by Grant 321-7291-BCT-0370 from the German Ministry for Research and Technology.

- Abelmann, W. H. (1973) *Annu. Rev. Med.* **24**, 145–152.
- Woodruff, J. F. (1980) *Am. J. Pathol.* **101**, 427–479.
- Johnson, R. A. & Palacios, I. (1982) *N. Engl. J. Med.* **307**, 1119–1126.
- Reyes, M. P. & Lerner, A. M. (1985) *Prog. Cardiovasc. Dis.* **27**, 373–394.
- Melnick, J. L., Wenner, H. A. & Phillips, C. A. (1979) in *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, eds. Lennette, E. H. & Schmidt, N. J. (Am. Public Health Assoc., Washington, DC), pp. 471–534.
- Dec, G. W., Jr., Palacios, I. F., Fallon, J. T., Aretz, H. T., Mills, J., Lee, D. C.-S. & Johnson, R. A. (1985) *N. Engl. J. Med.* **312**, 885–890.
- Kandolf, R. (1987) in *Coxsackieviruses—A General Update*, eds. Friedman, H. & Bendinelli, M. (Plenum, New York), in press.
- Gall, J. G. & Pardue, M. L. (1969) *Proc. Natl. Acad. Sci. USA* **63**, 378–383.
- Wolf, H., zur Hausen, H. & Becker, V. (1973) *Nature (London) New Biol.* **244**, 245–247.
- Brahic, M. & Haase, A. T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6125–6129.
- Kandolf, R. & Hofschneider, P. H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4818–4822.
- Kandolf, R., Canu, A. & Hofschneider, P. H. (1985) *J. Mol. Cell. Cardiol.* **17**, 167–181.
- Kitamura, N., Semler, B. L., Rothberg, P. G., Larsen, G. R., Adler, C. J., Dorner, A. J., Emini, E. A., Hancak, R., Lee, J. J., van der Werf, S., Anderson, C. W. & Wimmer, E. (1981) *Nature (London)* **291**, 547–553.
- Racaniello, V. R. & Baltimore, D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4887–4891.
- Tracy, S., Liu, H.-L. & Chapman, N. M. (1985) *Virus Res.* **3**, 263–270.
- Lindberg, A. M., Stålhandske, P. O. K. & Pettersson, U. (1987) *Virology* **156**, 50–63.
- Iizuka, N., Kuge, S. & Nomoto, A. (1987) *Virology* **156**, 64–73.
- Hyypä, T., Stålhandske, P., Vainionpää, R. & Pettersson, U. (1984) *J. Clin. Microbiol.* **19**, 436–438.
- Tracy, S. (1984) *J. Gen. Virol.* **65**, 2167–2172.
- Rotbart, H. A., Levin, M. J., Villarreal, L. P., Tracy, S. M., Semler, B. L. & Wimmer, E. (1985) *J. Clin. Microbiol.* **22**, 220–224.
- Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
- Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
- Nelson, J. A., Fleckenstein, B., Galloway, D. A. & McDougall, J. K. (1982) *J. Virol.* **43**, 83–91.
- Rueckert, R. R. & Wimmer, E. (1984) *J. Virol.* **50**, 957–959.
- Godman, G. C. (1973) in *Ultrastructure of Animal Viruses and Bacteriophages*, eds. Dalton, A. J. & Haguenu, F. (Academic, New York), pp. 133–153.