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DETECTION OF TYPE-COMMON AND TYPE-SPECIFIC HERPES SIMPLEX VIRUS DNA SEQUENCES USING RNA PROBES

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ABSTRACT

A nucleic acid hybridization assay to identify clinical isolates of herpes simplex virus (HSV) was developed using in vitro synthesized, radioactively labelled RNA transcripts from virus-specific DNA fragments cloned in transcription vector pGEM3. RNA probes derived from the HSV-1 BamHI Q DNA fragment hybridized strongly with HSV-1 and HSV-2 DNA, while RNA derived from HSV-1, EcoRI I, HindIII - PvuII DNA fragment hybridized with HSV-1 DNA only. The results of HSV typing using both common and type-specific probes correlated well with monoclonal antibody typing and restriction endonuclease analysis. Neither probe bound to cytomegalovirus, Epstein-Barr virus, varicella zoster virus, or pseudorabies virus DNA.

INTRODUCTION

Various approaches have been used to rapidly detect herpes simplex virus (HSV) in clinical specimens. An enzyme-linked immunosorbent assay (ELISA) was sensitive and specific for detecting HSV antigens[1,2] but may not be applicable if the specimens also contain HSV-neutralizing antibodies.[3] Differentiation of HSV type 1 (HSV-1) from type 2 (HSV-2) has been achieved using type-specific monoclonal antibodies.[4,5] However, this approach may not identify all HSV isolates because of antigenic diversity found among

them.[4] Restriction endonuclease (RE) analysis of HSV DNA has been a definitive approach for distinguishing between HSV types.[6,7] However, this technique seems to be useful only in epidemiological investigations[8,9] and has not been widely used in clinical laboratories.

DNA–DNA nucleic acid hybridization using DNA probes that recognize both HSV–1 and HSV–2 have been used successfully.[10-13] In these assays, differentiation of the virus types was based on binding capacity,[12] rate of hybridization,[10] or the stringency of the washing conditions.[13] However, these DNA probes can not be used at concentrations higher than 10^6 cpm per ml because of unacceptional background levels.[14,15] To avoid this and to increase the hybridization efficiency, RNA probes have been employed in many DNA and RNA virus detection procedures.[15-18] These probes were generated from transcription vectors containing DNA or cDNA fragments, respectively. The sensitivity of detection using RNA probes was shown to be almost tenfold higher than probes prepared from nick–translated DNA of the same length.[14,19] In addition, RNA probes could be used at higher concentrations than DNA probes without producing hybridization background.[14,15]

Since HSV–1 *Bam*HI Q fragment contains the thymidine kinase *(tk)* gene which is approximately 80% homologous to that of HSV–2's,[20] it may be a good type–common probe. For construction of a type–specific probe, DNA fragments larger than 1 kb may not be unique for either HSV–1 or HSV–2 because the herpes simplex viruses share 40% of their base sequences.[21] However, Draper *et al*[22] recently demonstrated that a DNA fragment located between 0.647 and 0.653 map units of HSV–1 contained few or no homologous sequence to HSV–2 *Bql* II I fragment. In this communication, we present our use of T7–generated, type–common and type–specific RNA probes for definitive identification and typing of HSV isolates from the clinical specimens. A comparison of this technique with fluorescent monoclonal antibody staining of infected cells and RE analysis of HSV DNA has also been described.

MATERIALS AND METHODS

Cells and Viruses

Vero cells for the isolation and propagation of HSV were cultured in growth medium composed of M199 with Earle's salts supplemented with 10% heat–inactivated fetal bovine serum (FBS; GIBCO, New Zealand). The amount of FBS was reduced to 1% in maintenance medium.

HSV isolates were obtained from patients with genital herpes treated at the sexually–transmitted disease clinic, Bangrak Hospital, Bangkok, Thailand. Each virus isolate was passaged 2–4 times in Vero cells before use. HSV–1 (KOS) and HSV–2 (Baylor 186) were originally provided by Dr. Chantapong Wasi of the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The HSV–1 ATCC

VR 733 of the American Type Tissue collection (Rockville, MD, U.S.A.), the AD-169 strain of cytomegalovirus (CMV), the Epstein–Barr virus (EBV) from secreting cell line (Ramos) of the European Collection of Animal Cell Culture (Porton Down, Salisbury, Wiltshire, England) and the pseudorabies virus (PR), isolated in 1978 from the brain of a fatal piglet in Nakorn Pathom province, Thailand, were maintained by Ms. Pranee Sithisarn of the Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. Varicella–zoster virus (VZV) was kindly provided by Dr. Boondee Atikij of the Department of Medical Science, National Institute of Health, Nonthaburi, Thailand.

Preparation of viral DNA

Each isolate was inoculated into 16 oz–tissue culture bottle containing a confluent monolayer of Vero cells at approximately 1–5 plaque forming unit per cell and allowed to adsorb for 1 hour after which maintenance medium was added. The infected cells were harvested usually in 18–24 hours at 37°C when they showed maximal cytopathic effect. HSV DNA was obtained, free from host cellular DNA, by Triton–NaCl extraction.[23,24] Briefly, one volume of an infected cell suspension (approximately 1.5×10^7 cells per ml) was mixed with 9 volumes of lysing solution (0.25% Triton X–100, 10 mM EDTA, 10 mM Tris–HCl, pH 7.9) and incubated at room temperature for 10 minutes with gentle mixing. NaCl was then added to a final concentration of 0.2 M and centrifuged at 1,000×g for 10 minutes at 4°C. The supernatant was carefully decanted and treated with RNaseI (50 μg per ml for 2 hours at 37°C) and proteinase K (100 μg per ml in 1% SDS for 2 hours at 37°C). DNA was clarified by two cycles of phenol–chloroform extractions and a chloroform extraction, ethanol precipitation, and then resuspended in 10mM Tris–HCl, 2 mM EDTA (TE), pH 7.5. All other viruses, in either infected cells or cell lysates were treated with a lysing solution (0.4 M Tris–HCl, pH 8.0, 100 mM EDTA, 1% SDS and 200 μg/ml proteinase K) for 1-2 hours at 50°C–60°C and extracted with an equal volume of phenol–chloroform followed by extraction with chloroform only. DNA denaturation was performed by the addition of 0.1 volume of 3.0 M NaOH and subsequent incubation for 30 minutes – 1 hour at 60°C–70°C. The preparations were neutralized by the addition of one volume of 2 M ice–cold ammonium acetate, pH 7.0.

DNA blots

All DNA samples were blotted onto nitrocellulose filter (BA 85, Schleicher & Schuell, Keene, NH) using either a Slot Blotter (Schleicher & Schuell) or a Hybridot^R Manifold (Bethesda Research Laboratories, BRL, Gaithersburh, MD, U.S.A.). The filter was soaked in 10X SSC (1X SSC contains 0.15 M NaCl and 0.015 M sodium citrate) for 10 minutes before use. The denatured samples were loaded into wells as slots or dots and the vacuum applied. After all samples had gone through the filters, each slot was washed with 10X SSC. The filter were blotted, dried under a heat lamp and baked for 2 hours at 80°C in a vacuum oven. Dried filters were stored at 4°C until use.

Preparation of probes

All restriction enzymes were obtained from Bethesda Research Laboratory. Reactions were performed in the buffers recommended by the manufacturer for 1 hour at 37°C, using 1 unit of the enzyme/μg of DNA. Digested DNA samples were analyzed by agarose gel electrophoresis.[25]

The HSV-1 *Bam*HI Q[26] (~3.5 kb, Fig. 1A) and *Eco*RI I[27,28] (~13.1 kb; Fig. 1B) fragments obtained after restriction enzyme digestion were purified using 1% agarose (low melting point) gel electrophoresis[29] and inserted into the pGEM3 plasmid (Promega Biotec, Madison, WI, U.S.A). Before use, this vector, containing the promotor site for SP6 and T7 RNA polymerases, was restricted with the corresponding enzymes, treated with 1 unit of alkaline phosphatase in CIP buffer (50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine) for 1 hour at 37°C, repurified using phenol-chloroform extraction followed by ammonium acetate-ethanol precipitation. The recombinant plasmids were used to transform the DH5 α strain of *Escherichia coli* as described by Lederberg and Cohen.[30]

Insert-positive bacterial clones were identified on agar plates containing 100 μg ampicillin per ml by colony hybridization[25] using radiolabelled (nick translated) insert DNA, and expanded in LB broth containing the same amount of ampicillin. Plasmid DNA samples were extracted using the method described by Birnboim and Doly,[31] and the size and orientation of the insert DNAs were determined by RE analysis.

The pGEM3 containing HSV-1 *Bam*HI Q fragment was designated as pWC1 and purposed for use as a common probe, while the pGEM3 carrying HSV-1 *Eco*RI I fragment containing the HSV-1 specific sequence was further subcloned. The latter plasmid was primarily freed from high molecular weight bacterial DNA using the polyethylene glycol method proposed by Krieg and Melton,[32] followed by double digestion with *Hind*III and *Pvu*II restriction enzymes. The DNA was blunt-ended using the Klenow fragment of *E. coli* DNA polymerasel and modified with *Eco*RI linkers (BRL).[25] Cohesive ends of this fragment were generated by cutting with an excess of *Eco*RI, purified by phenol-chloroform extraction and ammonium acetate-ethanol precipitation. The DNA was resuspended and ligated to dephosphorylated (*Eco*RI restricted) pGEM3. The insert-positive clones were obtained as described previously. This pGEM3 containing an HSV-1 *Hind*III - *Pvu*II (~730 bp, Fig. 1B) fragment was designated as pWC28 and intended for use as an HSV-1 specific probe.

Radiolabelled DNA probes were prepared using ^{32}P-deoxycytidine-5'-triphosphate (800 Ci/mM) and nick translation kits supplied by New England Nuclear, Boston, U.S.A.[33] The probes were purified, using Sephadex G-50 by the spun column method.[25]

To prepare radiolabelled RNA probes, insert-positive transcription plasmid DNA was prepared using the method of Krieg and Melton.[32] Both plasmids were linearized with *Hind*III restriction enzyme and purified by phenol-chloroform extraction and ammonium

acetate–ethanol precipitation. High specific activity radiolabelled RNA was prepared by adding 1 μg of template DNA and 20 units of T7 RNA polymerase into a reaction mixture containing 40 units of RNAsin, 10 mM dithiothreitol (DTT), 50 μCi ^{32}P–Uridine–5′–triphosphate (800 Ci/mM; New England Nuclear), 500 μM cold ribonucleotide triphosphates (ATP, CTP and GTP), and 12 μM uridine 5′ triphosphate in transcription buffer supplied by the manufacturer (Promega Biotec, Madison, WI). After incubation for 1 hour at 37°C, the DNA template was destroyed by treatment with 1 unit of RQ1 DNAase for 15 minutes at 37°C. The radiolabelled RNA was purified by phenol–chloroform extraction and ammonium acetate–ethanol precipitation.

The procedure described by Miller[34] was used to determine the actual sizes of RNA probes generated by pWC1 and pWC28; the molecular weight standards RNA Ladder were from Bethesda Research Laboratory. The samples were loaded on 1.5% formaldehyde agarose gel, electrophoresed, vacuum –dried and exposed to an X–ray film.

Hybridization procedure

For DNA–DNA hybridization, baked filters were prehybridized for 2 hours at 42°C in heat–sealed bags with prehybridization buffer (5X NET;20X NET contains 3.0 M NaCl, 0.3 M Tris, pH 7.5, and 20 mM EDTA; 5X Denhardt's solution, 100 μg/ml denatured Salmon sperm DNA and 0.5% SDS). Thereafter, the prehybridization solution was replaced with a hybridization buffer: (5X SSC, 1X Denhardt's solution, 20 mM NaH_2PO_4, pH 6.5, 50% formamide, 100 μg/ml denatured Salmon sperm DNA, 10% dextran sulfate), containing 10^7 cpm per ml of denatured ^{32}P–nicked–translated DNA probe. The reaction was carried out for 16 hours at 42°C.

For DNA–RNA hybridization, the oven–dried nitrocellulose papers were treated with a prehybridization buffer (6X SSC, 2% SDS, 5X Denhardt's solution, 100 μg/ml of denatured salmon sperm DNA and 50 μg/ml of heat–denatured tRNA) for 2 hours in heat–sealed bags at 68°C. The solution was replaced with a fresh hybridization buffer containing 10^6 cpm per ml of ^{32}P–labelled RNA probe, which had been boiled for 5 minutes, and incubated for an additional 16 hours at 68°C.

Filters were then washed twice in 2X SSPE (1X SSPE contains 0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl and 1 mM EDTA) and 1% SDS for 10 minutes each at room temperature and 4 times in 2X SSPE and 0.1% SDS at either 28°C (non–stringent washing conditions) or 65°C (relatively stringent washing conditions). Bound radioactivity was detected by exposure to Fuji X–ray film at –70°C before processing.

Typing of HSV

An immunofluorescence assay using monoclonal antibody (TC–FA) and/or RE analysis of viral DNA have been used for typing clinical HSV isolates in our laboratory. Type–specific monoclonal antibodies used in TC–FA were obtained from Kallestad, Austin,

TX, U.S.A. Briefly, isolates were grown in Vero cells on cover slips in Leighton tubes. When the infection reached 2^+ to 3^+ CPE, the coverslips were removed and processed using guidelines provided by the company. The slides were examined immediately using a fluorescence microscope.

Restriction endonuclease analysis of purified HSV DNA derived from these isolates was carried out essentially as described by Maniatis *et al*[25] using *Bam*HI, *Eco*RI, and *Kpn*I restriction enzymes. Differentiation between HSV–1 and HSV–2 was accomplished by analysis of their electrophoretic profiles on 0.7% agarose gels.

RESULTS

Specificity and sensitivity of DNA and RNA probes

Transcription products produced using pWC1 and pWC28 were characterized by electrophoretic analysis. Most of the synthesized RNA using either template was heterogeneous in character. Transcripts from pWC1 had sizes in the range 3 kb to 0.4 kb and those from pWC28 were generally less than 1 kb (Fig. 2).

The virus specificities of the nucleic acid probes was tested using stringent or non–stringent hybridization washing conditions. None of the radiolabeled probes reacted with a panel of related herpesviruses including CMV, EBV, VZV or PR viruses (data not shown). Representative results using herpes simplex viruses are shown in Figure 3. The RNA probe generated using pWC28 hybridized strongly with HSV–1 at stringent washing conditions; however, a faint band of cross–hybridization was observed when relatively non–stringent conditions were employed. Using DNA probes generated from the nicked–translated whole plasmid, pWC1 hybridized with HSV–2 DNA to relatively lower degree than with HSV–1 DNA (Fig. 3D) and pWC28 gave a weak band even though stringent conditions were used. This suggests a cross–hybridization with HSV–2 DNA.

The sensitivity of these probes was determined. Both RNA probes could detect about 20 pg of the whole plasmid DNA corresponding to about 11 pg WC1 and 4 pg WC28 insert DNAs or 3×10^6 and 5×10^6 HSV genome, respectively, after 16 hours of film exposure time (Fig. 4) and about 10 pg ($1.5-2.5\times10^6$ genome) after a 3–day film exposure (data not included). The limit of detection by nick–translated DNA probes was lower. Even though the amounts of RNA probes used was 10–fold lower than those of the DNA probes, they could detect less than 1 ng of their whole plasmid DNA while both DNA probes detected approximately 10 ng, after 2 hours of film exposure time (Fig. 5).

Use of RNA probes for typing HSV isolates

Since RNA probes generated from pWC1 and pWC28 could recognize the type–common and HSV–1 specific sequences, respectively, it was of interest to determine if these could be used for typing HSV isolates. Then local HSV isolates, the HSV–1 (KOS), and another HSV–1 prototype were tested in the DNA–RNA hybridization assay. Although

we did not include the prototype HSV–2 which showed no hybridization with pWC28 generated probe (Fig. 3), the results of typing of isolates were in agreement with the TC–FA and the RE analysis determined earlier (Fig. 6). Thus, these probes could discriminate between HSV–1 and HSV–2.

DISCUSSION

Typing of HSV isolates may provide valuable diagnostic and epidemiological data to supplement prognostic and treatment information. Since it was reported that, some antiviral agents exert different antiviral activity against both types.[35] In addition, the differential diagnosis of a primary genital infection caused by HSV–1 versus that caused by HSV–2 afforded prognosis.[36]

In the present study, we report for the first time the use of T7 RNA polymerase synthesized probes for specific and rapid identification of HSV isolates. The RNA probes were prepared full length corresponding to the size of the templates (Fig. 2). Thus, transcription by the T7 RNA polymerase could proceed to the end of template although the size of pWC1 which is larger than 2 kb might not be suitable for the preparation of a complete transcript.[37] These results also suggest that a type–specific RNA probe generated from transcription of pWC28 could discriminate between types of HSV since it hybridized with HSV–1 but not with HSV–2 when relatively stringent washing conditions were employed (Fig. 3B). It should be noted that, in general, RNA transcripts generated by run–off transcription using purified and linearized pGEM3 containing DNA fragments as templates are strictly homologous to integrated sequences. They do not contain any vector–specific sequences.

The cross–hybridization of DNA probes, obtained from nick–translation of pWC28, even at relatively stringent washing conditions, was observed (Fig. 3E). It can be explained by the sequence homologies between HSV–1 and HSV–2 which are scattering along the genome.[38] Thus, this DNA probe may not be suitable for specific HSV typing.

The sensitivity of RNA probes used in this study was relatively low compared to that observed by other investigators[15,16,18] who could detect viral RNA genomes in the clinical specimens at the level as low as 1 pg. This can be explained by the fact that RNA–RNA duplexes are more stable than DNA–RNA duplexes.[33] Thus, they still remained on the nitrocellulose filter after washing under highly stringent conditions while the less stable complexes would be washed off. However, both RNA probes were shown to be at least tenfold more sensitive than the nick-translated probes (Fig. 5). This is probably due to an efficient incorporation of radioactivity into RNA transcripts facilitated by the *in vitro* transcriptional step and since RNA probes are single stranded, there is no loss of radioactive activity because of reannealing.

The RNA probes which had been constructed from the prototype HSV–1 (KOS) strain hybridized efficiently with other HSV isolates (Fig. 6) ; these isolates differed to each

other and from the prototype strain based on RE analysis of their genome (manuscript in preparation). Although these isolates have been passaged in cell culture 2–4 times, the RE patterns were not altered.[40,41] It was found that both common and HSV–1 specific probes could distinctly identify the isolates according to their corresponding types determined previously by either TC–FA assay or RE analysis. RNA probes generated from pWC1 hybridized strongly to both HSV–1 and HSV–2 isolates, whereas the probe generated from pWC28 selectively hybridized to HSV–1 isolates only, using relatively stringent washing conditions.

Thus, DNA–RNA hybridization for the detection of HSV genomes described in the present study is specific and can be used for typing clinical HSV isolates. To increase the sensitivity and specificity of detection, the ideal probe should be prepared from a library of recombinant plasmids containing the HSV–specific fragments which span the entire genome. If this is done, the assay will be even more specific than the immunological assays which must rely on the specificities of polyclonal or monoclonal antibodies.

It has been known that the use of DNA probes in conjunction with the technique of target DNA amplification by the polymerase chain reaction (PCR)[42] enhances sensitivity of the detection method. However, we believe that with our more sensitive RNA probes when being used with the target DNA amplification technique similarly would even yield better results.

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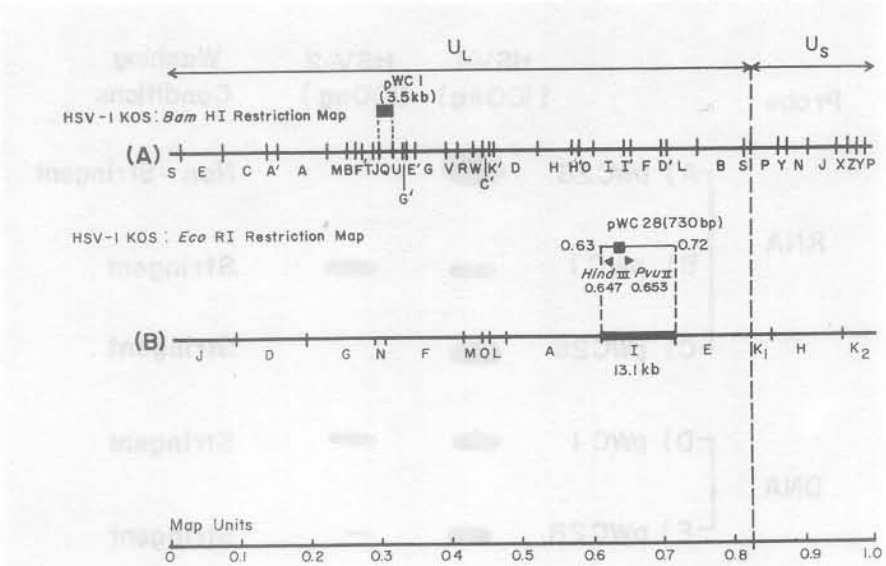

Fig. 1. Restriction endonuclease maps of HSV–1 KOS. A. *Bam*HI restriction map indicates locations of Q fragment (pWC1; ~ 3.5 kb) B. *Eco*RI restriction map of HSV–1 KOS indicates location of I fragment. An expanded map of this fragment illustrates location of *Hin*dIII – *Pvu*II cuts which yields a fragment of ~ 730kb (pWC28) is also shown.

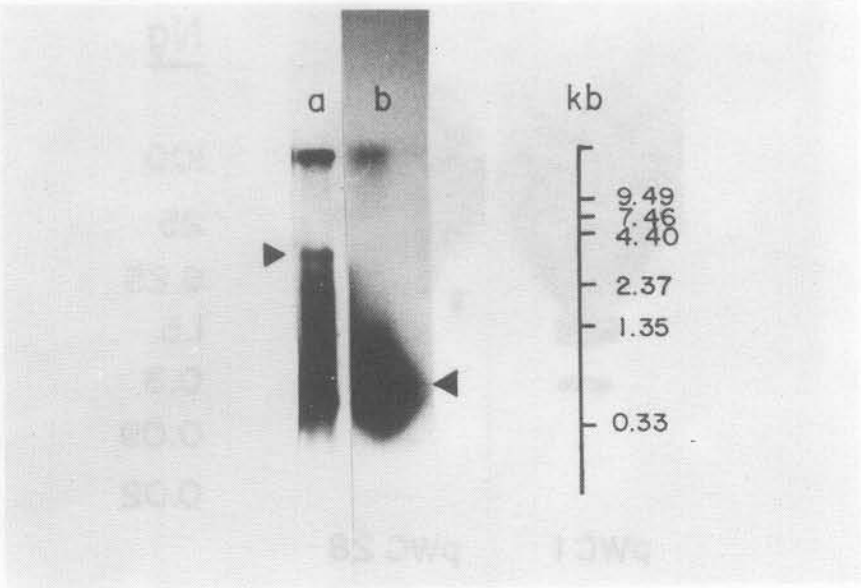

Fig. 2. Formaldehyde agarose gel electrophoresis of the products generated from transcription of linearized pWC1 (lane a) and pWC28 (lane b). The locations of fragment size markers in kb are also shown.

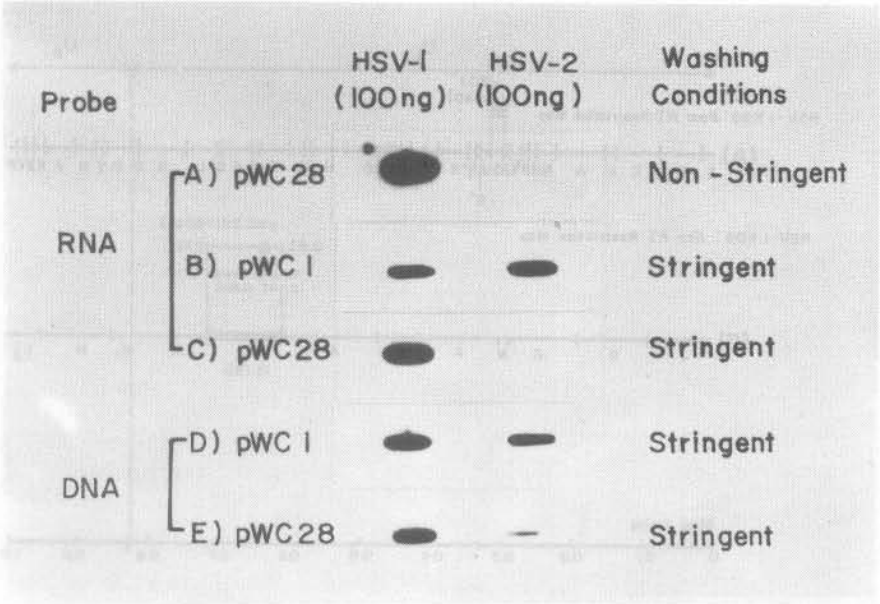

Fig. 3. Specificity of the DNA–RNA and DNA–DNA hybridization assays at non–stringent and relatively stringent washing conditions. A, hybridization using single–stranded RNA probe, washed at 28°C; B and C, hybridization using single–stranded RNA probes, washed at 65°C; D and E, hybridization using double–stranded DNA probes, washed at 65°C.

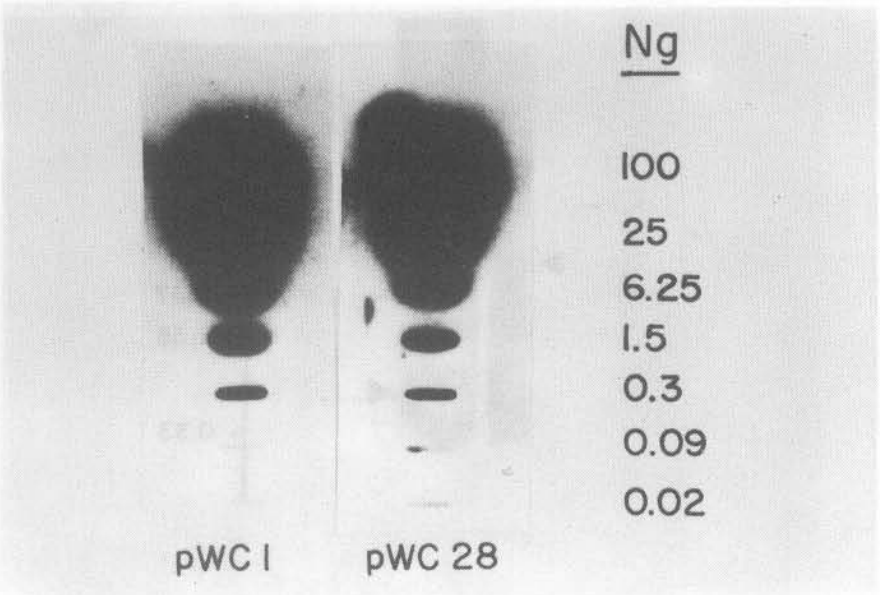

Fig. 4. Hybridization of single–stranded RNA probes with their homologous insert DNAs. The amount of probes used was 10^6 cpm per ml and X–ray film exposure time was 16 hours.

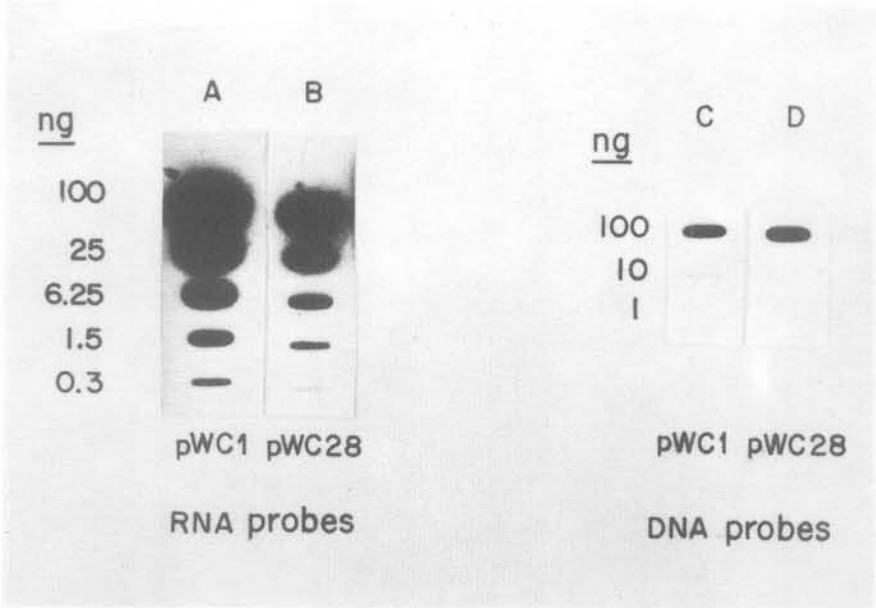

Fig. 5. The sensitivities of the single-stranded RNA and the double-stranded DNA probes in the hybridization assay with their homologous targets. The amounts of probes used were 10^6 cpm per ml (A and B) and 10^7 cpm per ml (C and D). The X-ray film exposure time was 2 hours.

Isolate	pWC1	pWC28	TC-FA[a]	RE analysis[b]
# 119	●		2	2
# 342	●		2	2
# 86	●		2	2
# 207	●		2	2
# 490	●		2	2
# 52	●		2	2
# 344	●	●	1	1
# 244	●		2	2
# 7	●		2	2
# 47	●		2	2
HSV-1(KOS)	●	●	1	1
HSV-I(ATCCVR 733)	●	●	1	1

Fig. 6. DNA-RNA hybridization, TC-FA and RE analysis of 10 HSV isolates. [a]A direct immunofluorescence technique using type-specific monoclonal antibodies.
[b]Purified HSV DNAs were digested with a panel of restriction endonucleases and analysed by electrophoresis.