Co-Localization of Two Different Viral Genomes in the Same Sample by Double-Chemiluminescence In Situ Hybridization

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ABSTRACT

A double-chemiluminescence in situ hybridization has been developed that combines the advantages of chemiluminescence with the detection of two different viral DNAs, i.e., herpes simplex virus (HSV) DNA and cytomegalovirus (CMV) DNA, in infected cells in the same specimen.

For the simultaneous detection of these two different viral DNAs, we used a biotinylated HSV DNA probe, which can be visualized by a streptavidin-horseradish peroxidase (HRP) complex amplified with biotinyl tyramide. This probe was followed by the use of a luminol-based chemiluminescent substrate for HRP and a digoxigenin-labeled CMV DNA probe visualized by anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (AP). This is followed by the detection with a dioxetane phosphate derivate as chemiluminescent substrate for AP. Since the final product of both chemiluminescent reactions was light emission, sequential images for the two hybridizations were taken and analyzed using a high-performance luminograph connected to an optical microscope and to a personal computer for image analysis. Positive signals for the presence of both HSV DNA and CMV DNA were noticed in infected cells in the same specimen with a sharp localization, absence of cross reactions and absence of background.

INTRODUCTION

In situ hybridization (ISH) techniques have been successfully used over the past decade to detect DNA or RNA sequences inside individual cells in a variety of molecular research and diagnostic applications. The need of increased sensitivity and of specific and quantitative analysis achieved from a digital imaging recently led to the developments of new methods such as chemiluminescence ISH, which can amplify ISH signals and give an image analysis of the results (7,8,15–17). Chemiluminescence ISH relies on the use of chemiluminescent substrates, which have been proposed as a more sensitive alternative to colorimetric substrates in various analytical techniques (2,5,9,13,14,21). In chemiluminescence ISH, labeled probes are visualized with a final enzymatic reaction involving either horseradish peroxidase (HRP) or alkaline phosphatase (AP) using highly sensitive chemiluminescent substrates. The spatial distribution of the steady-state light emitted from the hybridized probes is measured using a high-performance, low-light-level imaging luminograph connected to an optical microscope and to a personal computer (PC) for the quantification of the photon fluxes at a single photon level and for localization of the chemiluminescent emission inside individual cells.

Since previous works have demonstrated that chemiluminescence ISH is more sensitive than colorimetric staining and similar to ISH using radioactive probes (7,8,15–17), the aim of our work was to explore the use of chemiluminescence ISH for the simultaneous detection of multiple DNA sequences in the same sample. In fact, several authors have reported double-target ISH with colorimetric detection, utilizing both HRP and AP as reporter enzymes (4,6,10,18). In these cases, the two contrasting colors produced by the reactions could be clearly distinguished by bright-field microscopy. In this present study, we developed a double-chemiluminescence ISH that could combine the advantages of chemiluminescence with the detection of two viral DNAs, i.e., herpes simplex virus (HSV) DNA and cytomegalovirus (CMV) DNA, in infected cells in the same specimen.

MATERIALS AND METHODS

Samples

A reference laboratory strain (BE80) of HSV 2 and the Towne strain of human CMV were used at the multiplicity of infection (MOI) of 0.01 infectious units per cell (12) to infect HEL fibroblasts, which were harvested at the end of viral replication cycles (24 h post-infection [p.i.] for HSV and 72 h p.i. for CMV).

Harvested HSV- and CMV-infected cells were used to prepare: (*i*) slides with HSV-infected cells, (*ii*) slides with CMV-infected cells and (*iii*) slides with 50% HSV-infected cells and 50% CMV-infected cells. In these samples, since the MOI used for both viruses was 0.01 infectious units/cell, even noninfected cells were present.

As negative controls, slides with uninfected HEL cells were prepared.

For the hybridization assay, cells were smeared on glass slides pretreated as previously described (3) and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After fixation, cells were washed three times in PBS for 10 min each, then dehydrated with ethanol washes (30%, 60%, 80%, 95% and 100%) for 5 min each, air-dried and stored at 4°C until use.

Viral Probes

The HSV Type I and Type II DNA probe, which is commercially available (Enzo Biochem, New York, NY, USA), was biotinylated with the thymidine analogue 5[*N*-(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl] deoxyuridine triphosphate.

CMV DNA probe was constructed using the Towne CMV *Xba*I D fragment cloned in plasmid pACYC 184 and amplified in *E. coli* HB 101 (20). After large-scale preparation of plasmid, the D fragment was separated from the pACYC 184 vector.

Probe labeling was carried out by incorporation of digoxigenin-labeled deoxyuridine triphosphate (Dig-dUTP; Boehringer Mannheim, Mannheim, Germany) using the method of random-primed DNA labeling as previously described (4).

Double In Situ Hybridization

Pretreatment of cells. Cells were hydrated briefly in PBS and then placed in 0.02 *N* HCl for 10 min, then washed three times with PBS (5 min each). Cells were treated with 0.01% Triton® X-100 in PBS for 2 min and then washed three times with PBS (5 min each). Cells were treated with

pronase (0.5 mg/mL) in 0.05 M Tris-HCl buffer, pH 7.6, containing 5 mM EDTA for 5 min, then washed twice with PBS containing 2 mg/mL of glycine (3 min each). Cell monolayers were post-fixed with 4% paraformaldehyde in PBS for 5 min and washed twice with PBS containing 2 mg/mL of glycine (3 min each). Cells were then dehydrated by ethanol washes (30%, 60%, 80%, 95% and 100%).

Double-hybridization reaction. Dehydrated cells were overlaid with 20 µL of the hybridization mixture, which consisted of 50% deionized formamide, 10% dextran sulfate, 250 µg/mL of carrier calf thymus DNA, 24 ng/mL of biotin-labeled HSV DNA probe and 20 ng/mL of digoxigenin-labeled CMV DNA probe in 2× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). Cell samples and the hybridization mixture containing the labeled probes were denatured together by heating in a 92° ± 2°C water bath for 6 min, and then hybridized at 37°C for 3 h. After

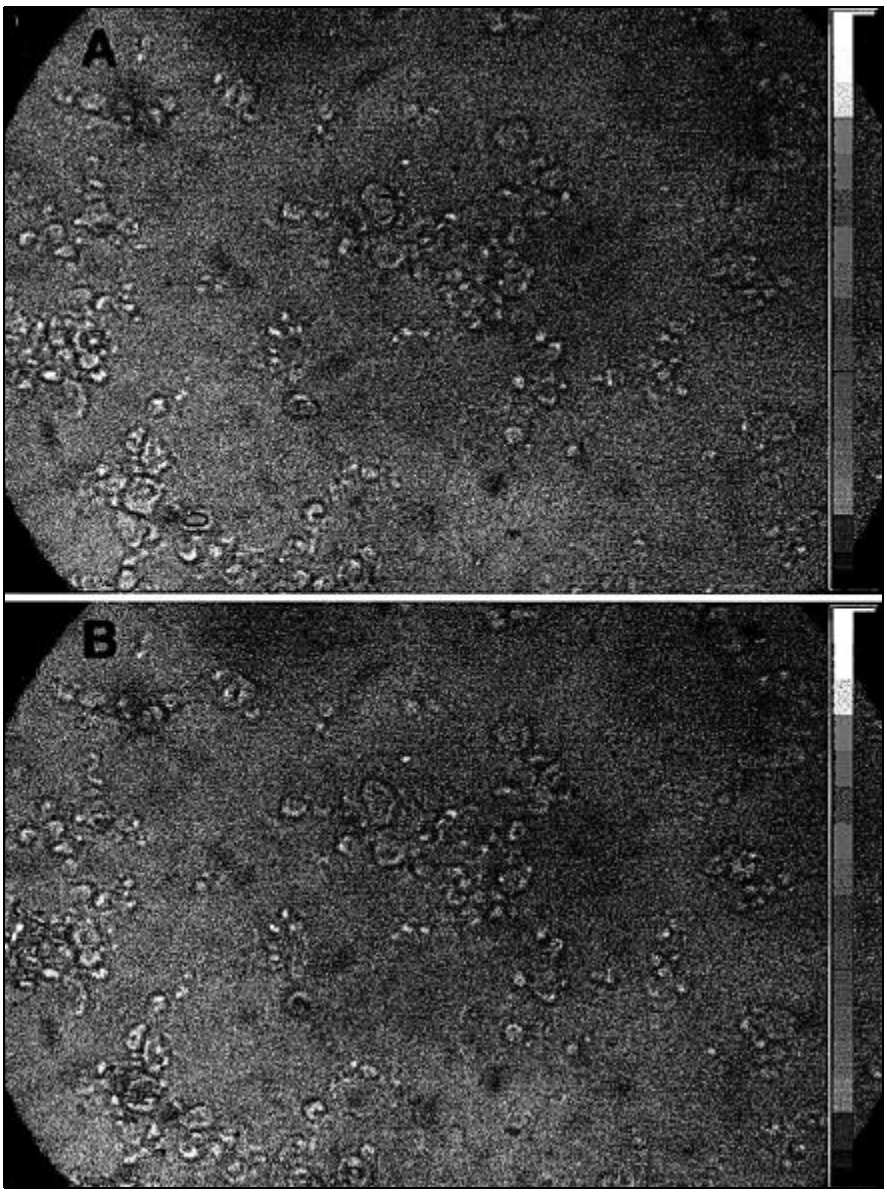

Figure 1. Chemiluminescence in situ hybridization revealing the presence of CMV DNA and HSV DNA in infected cells in the same specimen. From top to bottom: (A) luminescent signal processed with pseudo-color procedure derived from HSV DNA hybridization revealed with HRP/luminol-based reagent-reaction, (B) luminescent signal processed with pseudo-color procedure derived from CMV DNA hybridization revealed with AP/dioxetane reaction.

hybridization, cells were washed three times under stringent conditions (4).

Chemiluminescent detection of hybridized probes. Samples were washed 3 times for 5 min each in TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween® 20) at room temperature (RT) with gentle agitation. Cells were then incubated for 30 min at RT with a mixture of streptavidin-HRP complex (NEN Life Science Products, Boston, MA, USA) diluted 1:500 in Blocking buffer (Boehringer Mannheim) and polyclonal anti-digoxigenin Fab fragments conjugated to AP (diluted 1:500 in Blocking buffer). Cells were then washed three times for 5 min each in TNT buffer at RT.

For further amplification of peroxidase signal, the slides were incubated with biotinyl-tyramide (NEN Life Science Products) used at the optimized dilution of 1:50 in 1× amplification diluent (NEN Life Science Products) for 10 min at RT, followed by an incubation with streptavidin-HRP complex (diluted 1:100 in Blocking buffer) for 30 min at RT (19).

The optimal working dilution of each reagent used in the reactions was determined by preliminary block titration.

The chemiluminescent detections of the two probes were then performed sequentially; first for HSV DNA probe, which utilized HRP as a reporter enzyme, then for CMV DNA probe, which utilized AP as a reporter enzyme. Specifically, samples were washed 3 times in TNT buffer (5 min each), then treated with 40 µL of the luminol/H_2O_2/enhancer-based chemiluminescent reagent (ECL™; Amersham International plc, Little Chalfont, Bucks, England, UK) prepared following the manufacturer's instructions and measured for light output after a 2-min incubation. Cells were washed three times for 2 min each in Equilibration buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and then incubated at RT with 40 µL of undiluted adamantyl-1,2-dioxetane phenyl phosphate substrate (CDP-Star™; Tropix, Bedford, MA, USA) for 30 min. After having removed the substrate, cells were measured for light output.

The detection and analysis of the signals was performed using a high-performance, low-light-level imaging apparatus, (Luminograph LB-980; EG&G Berthold, Bad Wilbad, Germany), which permits emitted light measurement at a single-photon level. The video system consists of a high dynamic range pick-up tube 1″ (Saticon), which is a Vidicon-type tube with Se-As-Tl light target photoconductor, linked to an image intensifier by high-transmission lenses and combined with a videoamplifier. This system is connected to a Model BH-2 Optical

Microscope (Olympus Optical, Tokyo, Japan) and to a PC for quantitative image analysis. The microscope was enclosed in a dark box to prevent contact with the external light. The system operated in consecutive steps: (*i*) cells were recorded in transmitted light; (*ii*) the luminescent signals were measured sequentially for both enzymatic reactions with an optimized photon accumulation lasting 1 min with 2-s interval integration; and (*iii*) after a computer elaboration of the luminescent signal with pseudo-colors corresponding to the light intensity, an overlay of the images on the screen provided by the transmitted light and by the luminescent signals allowed the spatial distribution of the target analytes to be localized and evaluated. The light emission from each cell was quantified by defining a fixed area and summing the total number of photon fluxes from within this area.

Uninfected control cells that underwent a double-hybridization reaction were analyzed as described above, providing threshold background levels. A mean of 50 negative cells was analyzed for each run, and the average value of photon fluxes/s plus fivefold the standard deviation was considered the threshold for a positive signal (16,17). The net light signal was then calculated by subtracting the infected cell values with background values and expressed as integrated photons/s/area. Corrections for instrumental background and flat-field variations were automatically performed by the Model LB-980 Luminograph.

RESULTS AND DISCUSSION

As a first experiment to assess the sensitivity of our two probes detected by the chemiluminescent substrates, different dilutions of CMV and HSV DNAs (ranging from 2 pg to 0.5 fg) were dotted on nylon membranes and were hybridized with the respective probe. In regards to CMV DNA, 10 fg of homologous DNA could be detected as the end-point dilution. In dot-blot hybridization to detect HSV DNA, the most sensitive results were obtained after amplification of the signal generated by HRP using the tyramide system (19), reaching 50 fg of HSV DNA as end-point dilution.

To evaluate the specificity of the two probes and to avoid possible cross reactions, the presence of HSV DNA and of CMV DNA were analyzed with single chemiluminescence ISH, and positive signals were noted only when using the specific probe.

To assess the sensitivity of the chemiluminescent assay, we performed chemiluminescence ISH on latently infected cells containing a known number of viral genomic copies, [i.e., Caski cells, which contain 400–600 copies of human papillomavirus (HPV) DNA, and HeLa cells, which contain 10–50 copies of HPV DNA], using digoxigenin-labeled HPV probes (Kreatech, Amsterdam, The Netherlands) and the AP detection system. Chemiluminescent detection proved positive for both cell lines, and the luminescent signal changed in proportion to the known numbers of copies/cell; colorimetric detection proved positive for Caski cells but not for HeLa cells. These results were in accordance with a previous work (15).

After assessing the sensitivity and specificity of the two probes with their respective chemiluminescent detections and the sensitivity of the ISH assay, we evaluated the double-chemiluminescence ISH assay for HSV and CMV DNAs. The double-positive chemiluminescent reaction was detected sequentially: first for HSV DNA, revealed with HRP as detecting enzyme; then for CMV DNA, revealed with AP as detecting enzyme. Positive signals for the presence of both HSV DNA and CMV DNA were noticed in infected cells with a distinct localization, absence of cross reactions and absence of background (Figure 1).

The absence of cross-reaction in our double hybridization could be due not only to the specificity of the probes but also to the fact that the CMV DNA probe constructed in our laboratory was excised from the pACYC 184 vector, and this avoided hybridization of vector

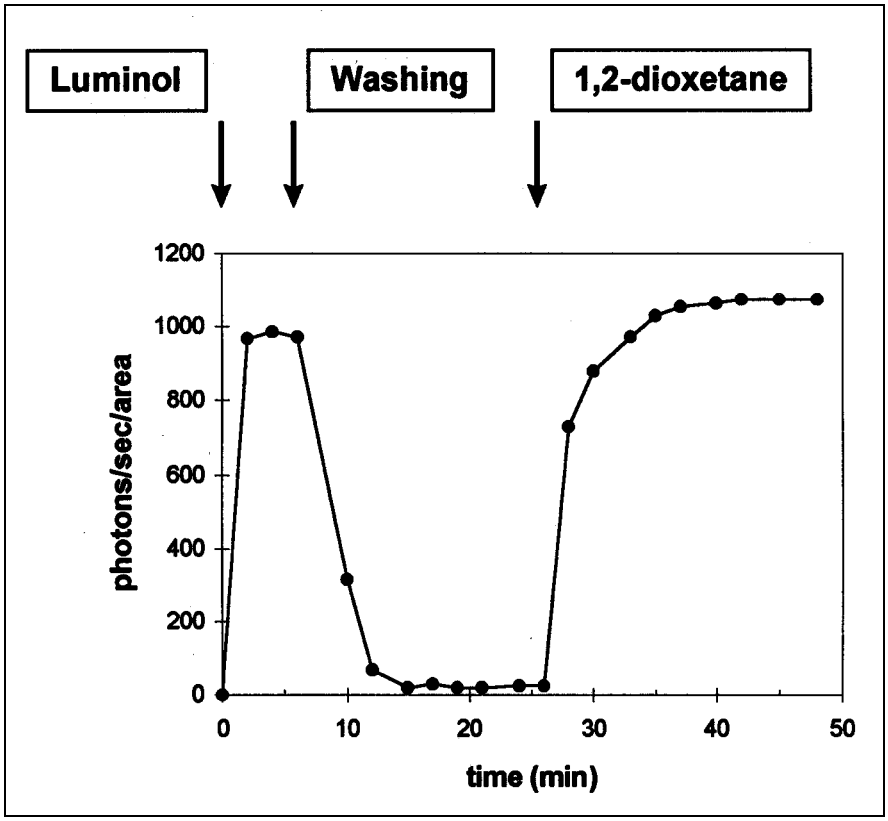

Figure 2. Chemiluminescent reactions in the double-hybridization assay. HRP/luminol-based reagent-reaction rapidly reaches a steady-state light output, which is maintained for a short time.Washings are then performed followed by AP/dioxetane reaction, which slowly reaches the steady-state light emission, which then lasts for a relatively long time. Measurements are taken during the steady-state light emissions.

sequences present in different probes. Moreover, the use of streptavidin-HRP complex and of anti-digoxigenin Fab fragments conjugated with AP avoided any possible nonspecific reactions due to the binding of Fc fragments of antibody to Fc receptors present in both HSV- and CMV-infected cells.

The specificity of our double-chemiluminescence ISH was confirmed by the following experiments: (*i*) No chemiluminescent-positive signal was revealed when HSV-infected cells were singularly hybridized with digoxigenin-labeled CMV DNA probe and, similarly, no positive signal was detected after hybridization of CMV-infected cells with biotinylated HSV DNA probe. (*ii*) HSV- and CMV-infected cells were completely negative after double hybridization with unlabeled HSV and CMV DNA probes. (*iii*) No chemiluminescent signal was observed when HSV- and CMV-infected cells were treated with the labeled probes and with the chemiluminescent substrates omitting the incubation with HRP and AP-conjugated detector systems. (*iv*) No positive signals were revealed when mock-infected fibroblasts were double-hybridized with labeled HSV and CMV DNA probes.

In our double-chemiluminescence reaction, the enzymatic reaction for HRP was usually performed before that of AP. This was done since the HRP/luminol-based reagent-reaction rapidly reaches a steady-state light output (after about 1 min), and this steady state is maintained for a short time (about 5–10 min), then the signal decreases while the AP/dioxetane reaction slowly reaches the steady-state light emission (after about 15 min), which then lasts for a relatively long time (at least 40 min). Short washes to remove the HRP substrate were thus sufficient to avoid any interfering photon emission during the AP reaction (Figure 2). Even to facilitate rapidity of the assay, the enzymatic reaction for HRP was usually performed before that of AP, consistent results were also obtained even when the substrate reaction sequence was reversed and longer washes were performed. In our assay, to optimize the light measurements, the signals were analyzed in the steady-state interval to ensure a linear relationship between the signal intensity and the enzyme activity. With our assay, since the positive signal was considered to be the one above threshold values obtained from negative samples, an objective evaluation of the results could be achieved.

Chemiluminescence ISH offers the capability to detect target sequences with great sensitivity (between 10–50 viral DNA copies/cell), thus proving more sensitive than colorimetric staining and similar to ISH using radioactive probes (7,8,15–17).

Moreover, chemiluminescence ISH is able to detect a higher number of positive cells/sample in comparison with colorimetric detection (16). Another advantage of chemiluminescence ISH is that the images obtained with chemiluminescence hybridization are stored in the PC as a permanent record of the reaction and can also be sent for evaluation or comparison to other laboratories using computer networks.

However, in comparison with other existing ISH methods to detect two targets, the chemiluminescence ISH has the limitation of requiring sequential staining and sequential signal visualization.

Our double-hybridization assay was performed on infected cell monolayers, but it can presumably be applied on tissue sections since our probes were successfully used in single-hybridization assays on frozen or formalin-fixed, paraffin-embedded tissue sections (1, 11). The double-chemiluminescence ISH developed in this work can thus be a useful tool for a sensitive and specific diagnosis of dual viral infections and for the detection and study of multiple specific genic sequences inside the cells. It can have further applications in pathology and virology laboratories undertaking research and/or routine diagnostics.

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