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Rapid Communication

A Novel In Situ Hybridization Signal Amplification Method Based on the Deposition of Biotinylated Tyramine[1]

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For amplification of in situ hybridization (ISH) signals, we describe a method using catalyzed reporter deposition (CARD). This amplification method is based on the deposition of biotinylated tyramine (BT) at the location of the DNA probe. The BT precipitate can then be visualized with fluorochrome- or enzyme-labeled avidin. Both for brightfield ISH (BRISH) and for fluorescence ISH (FISH), the detection limit was highly increased. This method is especially suitable for visualization of very weak ISH signals, such as those obtained by ISH using locus-specific DNA probes. Furthermore, CARD amplification of ISH signals (CARD–ISH) is highly sensitive, rapid, flexible, and easy to implement. Successful application of CARD–ISH with locus-specific DNA probes on histological and cytological samples may improve the determination of structural chromosomal aberrations in archival material. (*J Histochem Cytochem 43: 347–352, 1995*)

KEY WORDS: Fluorescence in situ hybridization; Brightfield in situ hybridization; Signal amplification; Metaphase spreads; Interphase nuclei; Tissue sections.

Introduction

During the last decade, in situ hybridization (ISH) techniques have become important tools to detect DNA target sequences (11,13,21). With these techniques, even very small DNA targets of 1–5 KB can be localized in metaphase spreads (6,16,30). To visualize small DNA targets by fluorescence in situ hybridization (FISH), high-sensitive charge-coupled device (CCD) cameras and image enhancement are necessary (19,28). Brightfield in situ hybridization (BRISH), using the horseradish peroxidase (HRP)-labeled avidin–biotin complex (ABC) technique (12), is applied routinely for demonstration of DNA targets in formalin-fixed, paraffin-embedded tissues (5,10, 26,27). However, only relatively large DNA targets with high repetitive sequences can be visualized by BRISH. For visualization of smaller DNA targets, the sensitivity of the ABC technique is too low, resulting in little or no detectable ISH signals.

For both FISH and BRISH, several methods have been described to amplify ISH signals. Some of these methods use modifications of the immunochemical detection of the probe label, such as silver intensification of diaminobenzidine (DAB) precipitate (18) or amplification of fluorescence signals according to the method of Pinkel et al. (20). Others are exploring the possibilities of amplifying the DNA targets by primed in situ extension (PRINS) (8,15) or polymerase chain reaction (PCR) in situ (7). Disadvantages of these amplification methods may be an increase in background staining, less target localization, and loss of morphology. A few years ago, a signal amplification method, catalyzed reporter deposition (CARD), and its application to immunoassays and immunohistochemistry were reported (1–3).

We describe here a method for amplification of ISH signals using CARD. First, DNA probe label is detected with a HRP-labeled detection system. Next, ISH signal amplification is achieved by deposition of biotinylated tyramine (BT) on the location of the probe label through the enzymatic action of HRP. Finally, the biotin source thus created is detected with fluorochrome- or enzyme-labeled avidin (Figure 1). This study shows that our ISH signal amplification method has an improved detection limit compared with standard ISH detection systems. Furthermore, this new approach can be applied in both brightfield and fluorescence ISH procedures. This is demonstrated by performing ISH with (a) a centromere-associated alphoid DNA probe specific for chromosome 3 (29) on formalin-fixed, paraffin-embedded tissue sections and (b) the locus-specific DNA probes HuαC (DNA target 16 KB) (22) and Bcl-2 (DNA target 2.8 KB) (23) on metaphase spreads and interphase nuclei.

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Materials and Methods

Cell and Tissue Samples. Metaphase spreads from human peripheral blood samples were prepared as described previously (4). Four-μm tissue sections of a formalin-fixed, paraffin-embedded transitional cell carcinoma of the urinary bladder were mounted on aminoalkylsilane (Sigma; St Louis, MO)-coated glass slides and heated overnight at 60°C. After dewaxing in xylene and rinsing in methanol, endogenous peroxidase activity was blocked for 20 min at room temperature (RT) in 1% H_2O_2–methanol. Slides were then rinsed in methanol and air-dried.

Pre-treatment of Samples. For good accessibility of target DNA to the probes, the specimens were permeabilized as described recently (14). Metaphase spreads were incubated for 10 min at 37°C in 100 μg (200 U) pepsin (Sigma) per ml 0.01 M HCl. Next, the slides were rinsed in demineralized water (MQ) and PBS and post-fixed in 1% formalin–PBS for 5 min at RT. After rinsing in PBS and MQ, the slides were dehydrated in an ascending alcohol series and air-dried. Paraffin tissue sections were incubated in 1 M NaSCN (Merck; Darmstadt, Germany) for 10 min at 80°C, followed by rinsing in MQ. Enzyme digestion was applied with 4 mg (8000 U) pepsin per ml 0.2 M HCl for 10 min at 37°C. Next, the slides were rinsed in MQ, dehydrated, and air-dried.

DNA Probes for ISH. Centromere-associated DNA probes for chromosomes 3 ($p\alpha3.5$) (29) and 12 ($p\alpha12H8$) (17) and DNA probes HuαC (16 KB target on chromosome 14q32) (22) and Bcl-2 (2.8 KB target on chromosome 18q21) (23) were labeled by nick-translation with biotin-14-dATP according to the suppliers' instructions (BRL; Gaithersburg, MD).

In Situ Hybridization. Centromere-associated DNA probe for chromosome 3 was hybridized to the paraffin tissue sections at a probe concentration of 2 ng/μl hybridization mixture containing 60% deionized formamide (Merck), 10% dextran sulfate (Sigma), 2 × SSC, pH 7.0 (SSC = 0.15 M NaCl, 0.015 M Na-citrate), and 50 ng/μl herring sperm DNA (Boehringer; Mannheim, Germany). Fifteen μl of probe mixture was applied to the tissue sections under a coverslip (18 × 18 mm) and sealed with rubber cement. Denaturation of probe and target DNA was performed by heating the slides in a moist chamber for 10 min at 80°C (10,21). ISH on metaphase spreads was performed as described by Wiegant et al. (30) with minor modifications. Briefly, target DNA in metaphase spreads was denatured for 3 min at 70°C in 70% formamide (Merck), 2 × SSC, pH 7.0. Next, slides were rinsed in 70% ethanol at −20°C, dehydrated, and air-dried. DNA probes HuαC and BCL-2 were used at a concentration of 2 ng/μl hybridization mixture. To compete out repeated sequences, a 250-fold excess of Cot 1 DNA (BRL) was added to the probe mix. After denaturation of the probe DNA for 3 min at 95°C, pre-annealing was performed for 30 min at 37°C. Probe mix (10 μl) was applied to separately denatured metaphase spreads under a coverslip and sealed with rubber cement. The centromere-associated DNA probe for chromosome 12 was applied on metaphase spreads at a probe concentration of 0.02 ng/μl hybridization mixture. Probe and target DNA were denatured simultaneously for 3 min at 70°C (21).

After hybridization overnight at 37°C, coverslips were removed by immersing the slides in 2 × SSC, pH 7.0, at 42°C. Post-hybridization washes (42°C) were carried out twice in 60% formamide (Merck), 2 × SSC, pH 7.0, for 5 min and twice in 2 × SSC, pH 7, for 5 min. Next, the slides were rinsed in PBS–0.05% Tween 20 (PBST; Merck).

Immunochemical Detection. For brightfield demonstration of biotinylated DNA probes in paraffin tissue sections, the HRP-labeled ABC method (12) was applied, using the Elite ABC kit (Vector Laboratories; Burlingame, CA). All incubation steps were performed in PBST containing 1% non-fat dry milk (PBSTM; Protifar, Nutricia, The Netherlands) for 30 min at 37°C. Washing steps between the incubations were three times in PBST for 5 min at RT. The tissue sections were pre-incubated with PBSTM for 15 min at 37°C. Subsequently, mouse anti-biotin (1:100; Dakopatts, Glostrup, Denmark), biotinylated horse anti-mouse (1:200, HsαM-bio; Vector) and ABC (avidin 1:100, biotin–peroxidase 1:100; Vector) were applied.

Visualization of the probe label was accomplished by precipitating diaminobenzidine (Sigma) through the reaction of HRP and H_2O_2 (Merck). Therefore, the sections were incubated with 0.05% diaminobenzidine-0.15% H_2O_2 in PBS–0.65% imidazole (Merck) for 5 min. After rinsing in MQ, the DAB signal was amplified with 0.5% $CuSO_4$ in 0.9% NaCl for 1 min and slides were again rinsed in MQ. Nuclei were counterstained with Mayer's hematoxylin. Finally, the samples were mounted in Permount (Fisher Scientific; Fair Lawn, NJ).

Fluorescence detection of the DNA probe for chromosome 12 and the locus-specific DNA probes HuαC and Bcl-2 was performed according to the amplification method of Pinkel et al. (20). All incubations were performed in 4 × SSC–0.05% Tween 20 (4SSCT; Merck) containing 1% nonfat dry milk (4SSCTM; Protifar) for 20 min at 37°C, and washing steps between the incubations, were three times in 4SSCTM for 5 min at RT. The metaphase spreads were pre-incubated with 4SSCTM for 10 min at 37°C. Next, the samples were incubated with fluorescein-labeled avidin DCS (1:500; Vector). The samples were incubated for two cycles with a biotinylated goat anti-avidin 1:100; Vector), followed by fluorescein-labeled avidin. After rinsing in 4SSCT, 4 × SSC, and MQ, the specimens were mounted in glycerol–Tris-HCl (9:1) containing Vectashield (1:4; Vector) and 4,6-diamino-2-phenylindole (DAPI, 1 μg/ml; Sigma).

Biotinylation of Tyramine. One hundred mg sulfosuccinimidyl-6-(biotinimide) hexanoate (NHS-LC–biotin) (Pierce; Rockford, IL) was dissolved in 40 ml 50 mM borate buffer, pH 8.0. Next, 30 mg tyramine-HCl (Sigma) was added. The solution was agitated overnight at RT and filtered. Final BT concentration was approximately 7 μM. Before application, BT was diluted in PBS.

ISH Signal Amplification. Before amplification of the BRISH signals, the ABC technique was performed, using MαB, HsαM-bio, and ABC. After rinsing in PBST and PBS, BT was precipitated through the enzymatic reaction of HRP. Therefore, the specimens were incubated with BT-PBS and 0.01% H_2O_2 for 5 min at RT. The BT concentrations were 0.007 μM for the DNA probe for chromosome 3 and 0.7 μM for the locus-specific DNA probes HuαC and Bcl-2. After rinsing in PBST, the biotin precipitate was detected with a second incubation of ABC (dilute ABC complex 1:4) or HRP-labeled avidin (1:50; Dakopatts) for 20 min at 37°C. After rinsing in PBST and PBS, HRP was visualized as described above.

For amplification of the FISH signals, first the ABC (HRP) method was performed. However, mouse anti-biotin was diluted $1:10^3$–$1:10^6$. BT precipitation was with BT-PBS (0.7 μM) and 0.01% H_2O_2 for 5 min at RT. After rinsing in 4SSCT, precipitated biotin was detected with fluorescein-labeled avidin DCS (1:1000; Vector).

Controls. The specificity of the centromere-associated DNA probes for chromosomes 3 and 12 was defined by FISH on metaphase spreads, resulting in specific signals on the centromeric regions at chromosomes 3 and 12.

To be sure that the observed ISH signals were specific, control experiments were performed; (a) with and without biotin label in the DNA probe and (b) with and without MαBio in the immunochemical detection. The sensitivity of the detection techniques was determined by dilution series of MαBio.

Evaluation of ISH Signals. For evaluation of the ISH signals, a Leitz DMRB/E microscope was used with the Wild 48/52 photoequipment (Leica; Wetzlar, Germany) for photomicrography. Fluorescence filterblocks A (BP340–380/RKP400/LP430) and 13 (BP450–490/RKP510/LP515) were used for detection of DAPI and fluorescein, respectively. Photomicrographs were made with Agfa APX25 (brightfield) and Kodak EPL 400 (fluorescence).
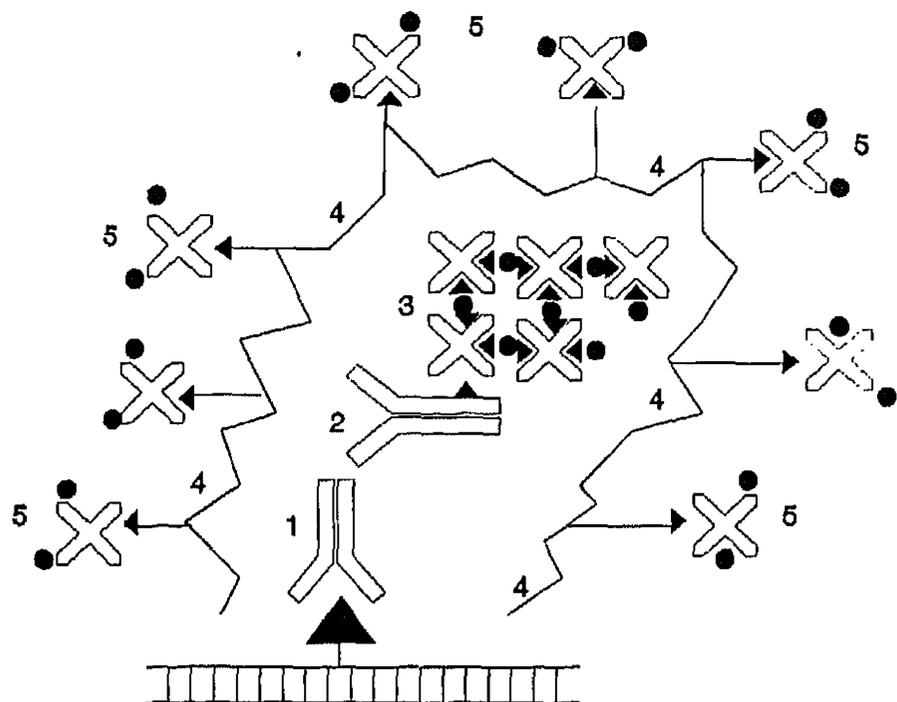
Figure 1. Schematic representation of the CARD-ISH technique. DNA probe label (▲) detection with (1) mouse anti-biotin, (2) biotinylated horse anti-mouse, and (3) avidin–biotin–peroxidase (ABC). Deposition of (4) biotinylated tyramine. Final detection with (5) fluorochrome- or enzyme-labeled avidin.

Results

Several combinations of DNA probes and DNA targets that had given unsatisfactory results with conventional ISH detection methods were tested. Initially, the CARD amplification system was applied in BRISH on formalin-fixed, paraffin-embedded tissue sections. Detection of the biotinylated centromere-associated DNA probe specific for chromosome 3 with the standard ABC (HRP) method resulted in small ISH signals (Figure 2A). Application of the ABC technique followed by the CARD method, as described by Adams (1) (BT 0.07 µM, 5 min), resulted in strong amplification of the ISH signals. However, the amplification was so excessive that most of the nuclei were covered with DAB precipitates (Figure 2B). The ISH signals interfered and could not be observed separately. The specificity of this reaction is shown in Figure 2C. Here, an unbiotinylated DNA probe was used in combination with CARD–ISH, showing no ISH signals in the nuclei. By diluting the primary antibody, mouse anti-biotin (MαBio), the ISH signals became smaller, but also more irregular in size. MαBio could be diluted 1:10⁴ before the signals disappeared. It was also possible to adjust the degree of amplification by varying the concentration and reaction time of BT. Good results were achieved with 0.007 µM BT incubation for 5 min at RT (Figure 2D). BT concentrations higher than 0.015 µM and incubation times longer than 5 min resulted in excessive amplification.

The utility of CARD amplification in FISH was also investigated. Centromere-associated DNA probe specific for chromosome 12 at a concentration of 20 pg/µl hybridization mixture was hybridized to metaphase spreads. The fluorescence detection system, using three layers of fluorescein-labeled avidin, resulted in small and weak ISH signals (Figure 3A). Applying more layers of avidin–fluorescein resulted in high background staining (results not shown). Detection of DNA probe label with the CARD amplification, using the BT concentration and incubation time as described above, was performed. Visualization of precipitated biotin was achieved with fluorescein-labeled avidin. However, the FISH signals were too weak for proper evaluation. Enhancement of the BT concentration (up
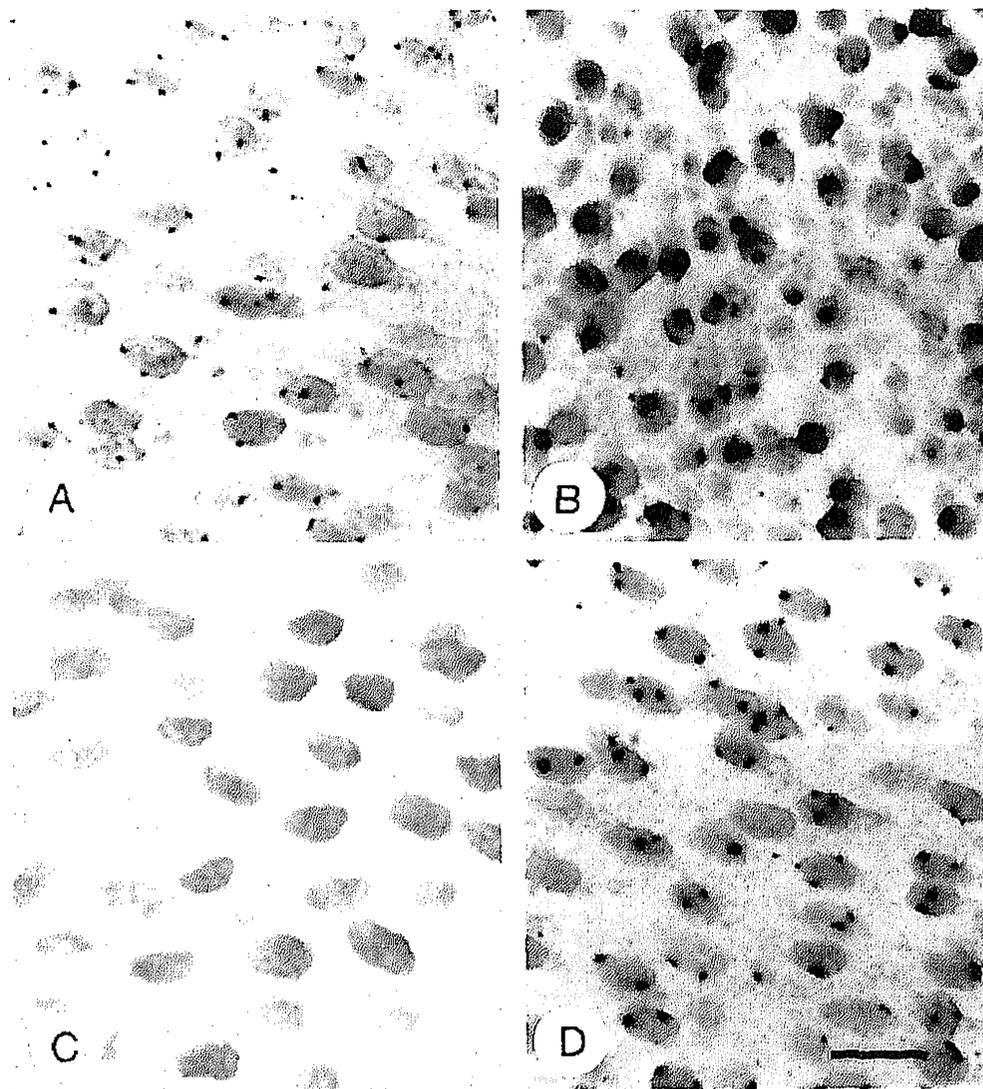

Figure 2. Results of CARD amplification in brightfield ISH on tissue sections. (A–D) ISH with a biotinylated DNA probe specific for the centromeric region of chromosome 3 on formalin-fixed, paraffin-embedded bladder tissue sections (4 µm). Visualization was achieved by (A) the standard ABC technique, (B) the CARD method with a BT concentration of 0.07 µM for 10 min, (C) the same as B but with the DNA probe without its biotin label, and (D) the CARD method with a BT concentration of 0.007 µM for 5 min. Original magnification × 400. Bar = 10 µm.

to 0.7 µM) resulted in very strong amplification of the FISH signals (Figure 3B). However, increased background staining was observed. Extending the BT incubation time (up to 20 min) also enlarged the FISH signals, but the signals became patchy, which hampered good localization. The amplification of the FISH signals could also be varied by diluting the primary antibody of the HRP detection system, i.e., MαBio. MαBio dilutions lower than 1:10³ resulted in large ISH signals but also high background staining due to a specific binding of MαBio to the specimen. In combination with 0.7 µM BT, the highest MαBio dilution used was 1:10⁶, resulting in still good detectable and evaluable FISH signals (Figure 3C). Even omitting MαBio resulted in specific FISH signals (data not shown). This can be explained by the fact that ABC can bind directly to the biotinylated DNA probe and thus accomplish signal amplification. Unbiotinylated probe DNA in the CARD–FISH procedure resulted in no ISH signal.

CARD–FISH was also applied for visualization of low-copy DNA sequences. We performed FISH with a biotinylated HuαC probe that recognizes a 16 KB target on chromosome segment 14q32. Detection with three layers of fluorescein-labeled avidin resulted in barely visible FISH signals, even using a ×100 (NA 1.3) oil objective lens. Applying CARD–FISH with the high BT concentration (0.7 µM) and short incubation time (5 min) resulted in a considerable amplification of the FISH signals, but probe localization was
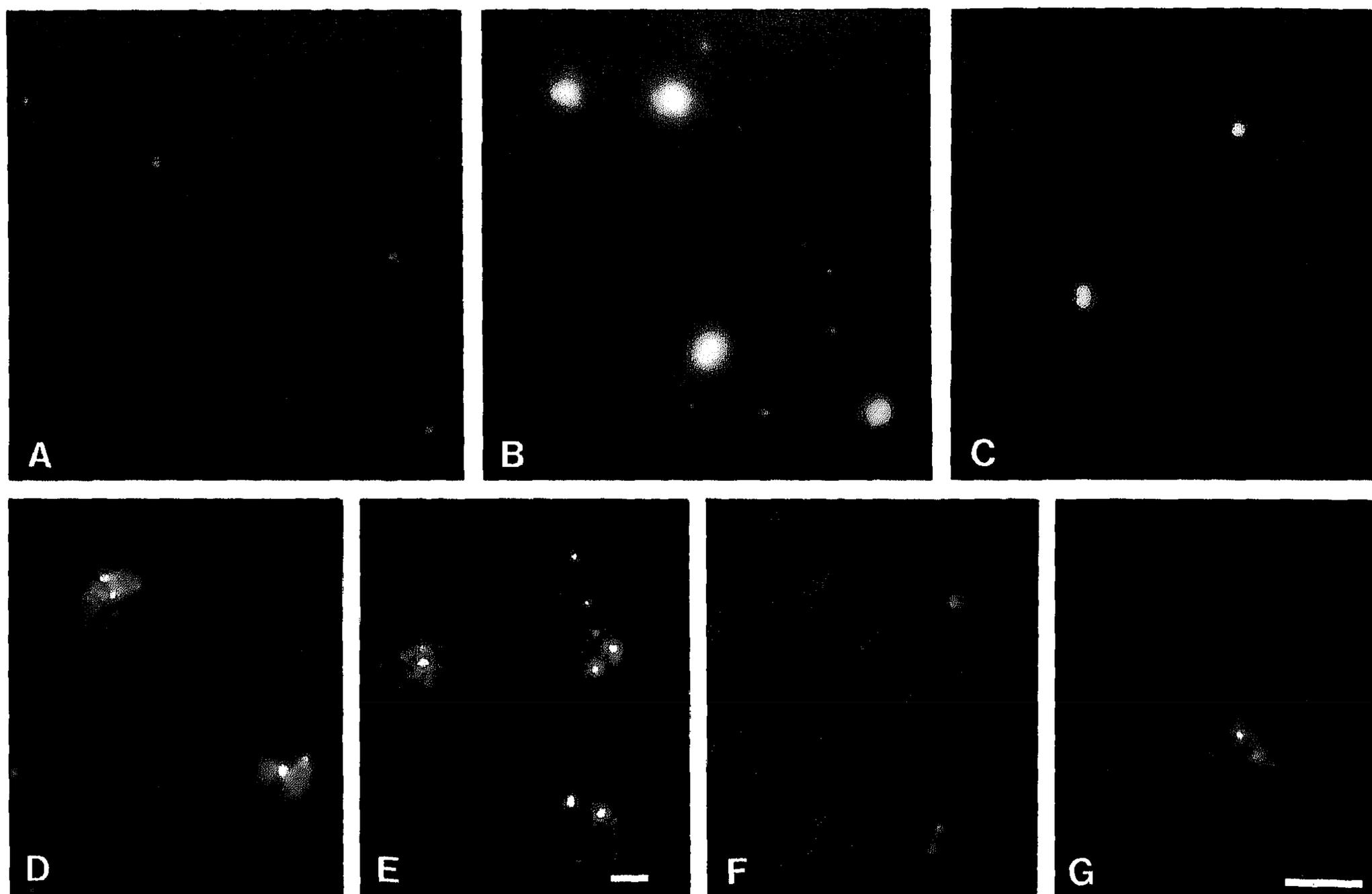
Figure 3. Results of CARD amplification in fluorescence ISH on metaphase spreads. (A-C) FISH with a DNA probe specific for the centromeric region of chromosome 12 (20 pg/μl hybridization mixture) on interphase nuclei. Visualization of the biotinylated DNA probe was achieved with (A) three layers of fluorescein-labeled avidin, (B) CARD-FISH with MαBio 1:10⁶ and 0.7 μM BT, and (C) CARD-FISH with MαBio 1:10⁶ and 0.07 μM BT. (D) Metaphase chromosomes and (E) interphase nuclei after CARD-FISH (BT 0.7 μM, 5 min) with an HuαC probe recognizing a 16 KB target on chromosome 14. (F) Metaphase chromosomes and (G) an interphase nucleus with FISH signals for Bcl-2 (2.8 KB target) after CARD-FISH (BT 0.7 μM, 5 min). Original magnifications: A-D,F,G × 1000; E × 400. Bars: A-D,F,G = 4 μm; E = 10 μm.

difficult. Improvement of localization was achieved by diluting Mα-Bio up to 1:10⁵. Figure 3D shows these FISH signals on the q arms of chromosome 14. By diluting MαBio 1:10³–1:10⁴, clear FISH signals in interphase nuclei could be observed even with a ×40 (NA 0.70) objective lens (Figure 3E). The Bcl-2 probe, which recognizes a DNA target of 2.8 KB on chromosome 18q21, could not be visualized by conventional FISH detection methods. However, FISH signals were visualized by the CARD–FISH technique. MαBio could be diluted up to 1:10³, giving reasonable signals on the metaphase chromosomes (Figure 3F) and small but clear FISH signals in the interphase nuclei (Figure 3G).

Finally, the possibility of detection of low-copy DNA sequences for brightfield microscopy with CARD-BRISH was investigated. Again, the DNA probes HuαC and Bcl-2 were used. The primary antibody MαBio 1:10³ was applied in combination with a BT concentration of 0.7 μM and incubation time of 5 min. For good localization of the ISH signals, the precipitated biotin was detected by HRP-labeled avidin instead of a second incubation with ABC. Figures 4A–4D show clear BRISH signals for HuαC and Bcl-2 on metaphase chromosomes and interphase nuclei. The 16 KB target

detected by the HuαC probe could be visualized even with a ×25 (NA 0.50) objective lens (Figure 4B).

Discussion

In situ hybridization (ISH) is a method for detection of DNA target sequences and is applied in a wide variety of routine and research studies (5,11,13,16,21,24–28,30). The application of avidin–biotin systems (9) for detection and amplification of ISH signals has become widespread (20,24). However, an increase in the sensitivity of detection systems is still needed to permit routine detection of low-copy-number DNA sequences. A few years ago, a signal amplification method, catalyzed reporter deposition (CARD), and its application to immunoassays and in histochemistry was described (1–3). In the present study we describe a new ISH procedure, in which this CARD amplification method is combined with ISH techniques. After detection of the DNA probe with the ABC (HRP) system, ISH signal amplification is achieved by deposition of biotinylated tyramine at the location of probe label through the enzymatic action of HRP. Visualization of the precipitated biotin sites
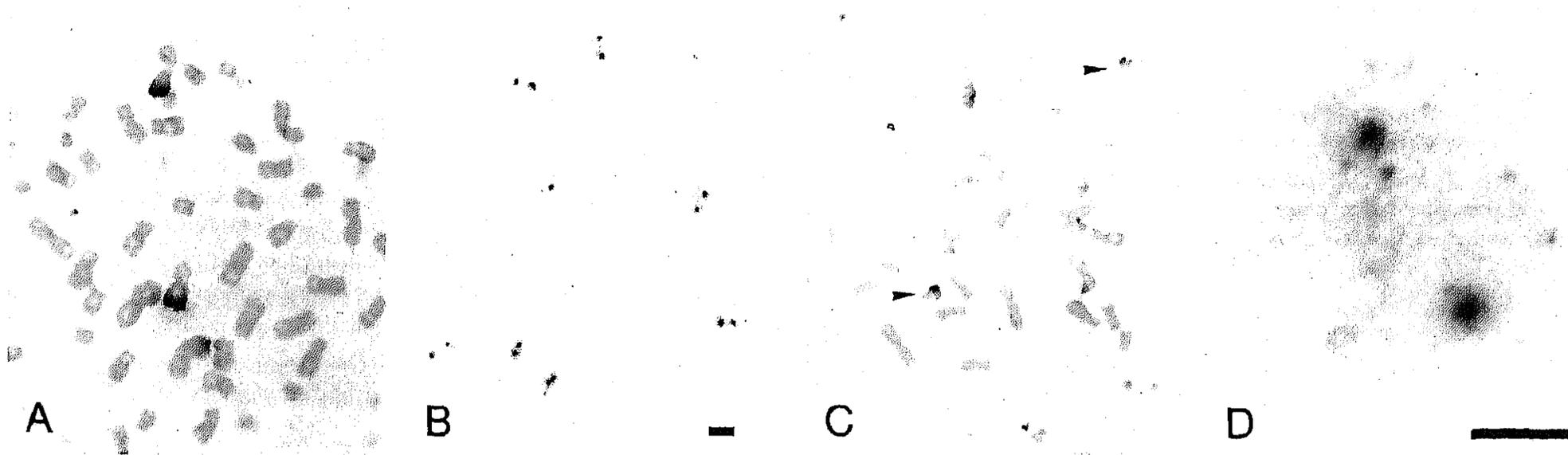
Figure 4. Results of CARD amplification in brightfield ISH on metaphase spreads. (A) Metaphase chromosomes and (B) interphase nuclei after CARD–BRISH (BT 0.7 µM, 5 min) with an HuαC probe that hybridizes with a 16 KB target sequence on chromosome 14. (C) Metaphase chromosomes (arrowheads) and (D) interphase nuclei with ISH signals for Bcl-2 on chromosome 18 (2.8 KB target) after CARD–BRISH (BT 0.7 µM, 5 min). Original magnification: A,C,D ×1000; B × 250. Bars: A,C,D = 4 µm; B = 15 µm.

was accomplished with fluorochrome- or enzyme-labeled avidin. The CARD–ISH technique proved to be a highly sensitive detection method compared with conventional detection methods.

Application of CARD amplification in FISH on metaphase spreads resulted in an increase of the FISH signals. A 16 KB DNA target sequence on chromosome 14q32 and a 2.8 KB DNA target sequence on chromosome 18q21 could be visualized by CARD–FISH in metaphase chromosomes and interphase nuclei. Moreover, the FISH signals could be observed easily by conventional fluorescence microscopy without the need for a CCD camera and image enhancement. The same single-copy DNA sequences could be demonstrated by CARD amplification in brightfield microscopy. The CARD–BRISH method demonstrated clear, well-localized ISH signals in metaphase chromosomes and interphase nuclei. At this time, experiments are being performed for determination of the smallest DNA target detectable by CARD–FISH. In addition, the usefulness of CARD–FISH for demonstration of oligonucleotide probes is being determined.

CARD–BRISH on paraffin tissue sections with the centromere-associated DNA probe specific for chromosome 3 demonstrated strong amplification of the ISH signals compared with the standard ISH technique. Therefore, CARD–BRISH will facilitate the evaluation of ISH results in tissue sections. With this method, a greater range of moderate and low repetitive DNA target sequences can be demonstrated in formalin-fixed, paraffin-embedded tissue sections.

It was possible to control the ISH signal amplification by adjusting the BT concentration and incubation time in combination with an appropriate MαBio dilution. Amplification of ISH signals in BRISH on paraffin tissue sections could be achieved by a low dilution of MαBio (1:10²) and a low concentration of BT (0.007 µM), providing at least a five-fold enhancement of the ISH signal size compared with standard ABC detection. Detection of short DNA target sequences with CARD–BRISH and CARD–FISH required higher BT concentrations. As a result of our experiments, we recommend a MαBio dilution of 1:10² and a BT concentration of 0.007 µM for detection of relatively large repetitive DNA target sequences in formalin-fixed, paraffin-embedded tissue sections. For detection of short, low-copy DNA sequences, a MαBio dilution of at least 1:10³ and a BT concentration of 0.7 µM is preferred.

Varying the dilution of the primary antibody (MαBio) can be used as a fine adjustment factor to obtain proper ISH signal sizes and a good signal-to-noise ratio, necessary for good evaluation. Particularly in CARD–ISH on metaphase spreads, owing to the high sensitivity of CARD–ISH, aspecific binding of MαBio resulted in background staining. To reduce this background staining and to obtain a good signal-to-noise ratio, the dilution of MαBio in CARD–ISH on metaphase spreads must be higher (at least 1:10³) than in conventional ISH techniques. The opportunity to accomplish variable ISH signal sizes and a good signal-to-noise ratio indicates that CARD–ISH is a very flexible technique for amplification of ISH signals.

At this time, we are investigating the application of CARD–BRISH for visualization of single-copy DNA targets on cytological and histological material. Recently, we described a double-target ISH technique for brightfield detection of two different DNA target sequences in one sample (14). Performing a double-target CARD–ISH procedure with both biotinylated tyramine and fluorescein-labeled tyramine (3) results in two different haptenized precipitates. The haptens biotin and fluorescein can be detected with labeled avidin and labeled anti-fluorescein, respectively. If this approach is successful, a wide variety of applications will be possible. For example, simultaneous detection of two different DNA loci in interphase nuclei will allow the study of structural chromosome aberrations, such as translocations, in brightfield microscopy.

In summary, we describe an ISH amplification technique that substantially increases the detection limit of ISH. This novel application of ISH signal amplification is highly sensitive, rapid, flexible, and easy to implement in both brightfield and fluorescence ISH procedures.

Acknowledgment

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