Amplification of gene fragments with very high G/C content: c7dGTP and the problem of visualizing the amplification products.

J Weiss, H D Zucht and W G Forssmann

Genome Res. 1994 4: 124-125

Amplification of Gene Fragments with Very High G/C Content: c^7dGTP and the Problem of Visualizing the Amplification Products

Johanna Weiss, Hans-Dieter Zucht, and Wolf-Georg Forssmann

Niedersächsisches Institut für Peptid-Forschung GmbH, D-30625 Hannover, Germany

PCR amplification of sequences from genomic DNA often yields multiple nonspecific bands, especially if the amplified regions are rich in guanosine and cytosine. G/C-rich sequences can cause hairpin loop structures that potentially give rise to problems for in vitro DNA amplification. In addition to this, G/C-rich primers are less specific because of a shorter stretch needed for recognition. Inclusion of the nucleotide analog 7′-deaza-2′-deoxyguanosine-5′-triphosphate (c^7dGTP) in the PCR reaction is the most suitable strategy that allows efficient amplification of the expected sequences.[1] First of all, PCR conditions should be optimized concerning the choice of primers, concentrations of all components, and cycling parameters. The primers used are the most critical parameter, determining success or failure of an amplification reaction.[2] Sometimes it is not possible to choose "optimal" primers, for example, because of a high G/C content of the investigated region. In this case, one should consider the application of c^7dGTP.

The base analog 7′-deaza-2′-deoxyguanosine precludes Hoogsteen bond formation, as the N-7 of the guanine ring is replaced by a methine.[1] The stability of Watson–Crick base-pairing is also reduced (ΔH: dG-dC base pair: −50 kJ/mole of stack/c^7dG-dC: −36 kJ/mole of stack.[3] Therefore, c^7dGTP reduces tertiary structure formation within the fragments and causes longer hybridizing stretches necessary for the chosen annealing temperature. This enhances specificity of the PCR. It has been shown that c^7dGTP can fully replace dGTP during PCR-amplification.[4]

However, the usage of c^7dGTP confronts the investigator with another problem: Fragments containing this analog can be stained only poorly with ethidium bromide. Normally, ethidium bromide fluorescence is enhanced if it intercalates into double-stranded DNA. The incorporation of c^7dGTP impairs ethidium bromide binding in the following way: The electron pair of N-7 purine nitrogens within the major groove of the DNA interacts with ethidium bromide. Because c^7dGTP lacks this nitrogen, the binding of ethidium bromide is reduced. Moreover, the decrease of N-7 nitrogen changes the dipole moment of the nucleotide, which alters adjacent base stacking and consequently the intercalation of ethidium bromide.[4] Therefore, the detection of DNA fragments containing c^7dGTP upon gel electrophoresis is difficult when using ethidium bromide staining. We tested silver staining as a method for visualizing those DNA fragments.

MATERIALS AND METHODS

The investigated gene (DRD4)[5] contains ~70% G/C. *Taq* DNA polymerase was purchased from Biomol (Hamburg, Germany).

Oligonucleotides

The oligonucleotides used as PCR primers were synthesized on an Applied Biosystems DNA synthesizer (model 394) and were purified using oligonucleotide purification cartridge P200003 (MWG-Biotech, Ebersberg, Germany). They were 24, 25, or 26 nucleotides long and had a G/C content between 64% and 73% [primer 1, 5′-CCCGCTCATGCTGCTGCTCTACTGG-3′: (sense); primer 2, 5′-GGTGGCACGTCGCGCCAAGCTGCAC-3′ (sense); primer 3, 5′-CTGTGCGACGCCCTCATGGCCATG-3′ (sense); primer 4, 5′-GATCTTGGCACGCCGCCTGCGGG-3′ (antisense); primer 5, 5′-TGCAGCTTGGCGCGACGTGCCACC-3′ (antisense)]. Used primer combinations are as follows: 1 (1/4), b (2/4), and c (3/5).

Preparation of Genomic DNA

Genomic DNA was purified by Qiagen Blood DNA Kit (Qiagen, Hilden, Germany).

Amplification Conditions

PCR amplification was carried out in 50-μl reactions containing *Taq* DNA polymerase reaction buffer (20mM Tris-HCl (pH 8.55), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 150 μg/ml of BSA), 0.5 μM of each primer, 200 μM each of dATP, dCTP, dTTP and c^7dGTP, 1 μg of genomic DNA, and 1.6 units of *Taq* DNA polymerase. Samples were first preheated for 4 min at 95°C to melt genomic DNA. Application of *Taq* DNA polymerase (hot start PCR) was followed by 39 cycles of 1 min at 94°C (denaturation), 35 sec at 62°C (annealing), and 1 min at 72°C (elongation) for primer combinations a and b. Using primers 3 and 5 (combination c), application of *Taq* DNA polymerase was followed by 35 cycles of 1 min at 98°C (de-

4:124–125©1994 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/94 $5.00

naturation) and 4 min at 70°C (annealing and elongation). In both cases, the run was terminated with a final elongation step of 10 min at 72°C. The samples were then stored at 4°C until needed.

Native Polyacrylamide Gel Electrophoresis

Fourteen microliters (for ethidium bromide staining) or 7 μl (for silver staining) of the PCR products was separated by nondenaturing 8% polyacrylamide gel electrophoresis in 1× TBE buffer (0.1 M Tris-HCl at pH 8.3, 85 mM boric acid, 1 mM EDTA).

Ethidium Bromide Staining

The gels were rinsed once in water for 10 min and stained with 1 mg/liter of ethidium bromide for 30 min. They were subsequently rinsed in water for an additional 15 min.

Silver Staining

The gels were rinsed twice in water for 10 min and stained with 0.2% $AgNO_3$ for 30 min. They were subsequently washed four times for 10 sec with water and developed with 0.4% formaldehyde in 1.5 M NaOH until the bands were seen clearly. The reaction was stopped by washing the gels twice with water containing 10% methanol and 10% acetic acid.

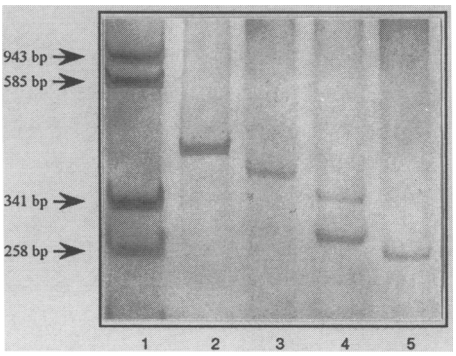

FIGURE 1 c^7dGTP containing PCR fragments of the DRD4 gene stained with silver nitrate. Aliquots of 7 μl of the PCR reaction mixtures were electrophoresed and stained with silver nitrate. (Lane *1*) 0.5 μg molecular weight marker pUC18 digested with *Sau*3A (sizes at *left*). (Lane *2*) 477-bp PCR fragment (primer combination c). (Lanes *3–5*) The DRD4 polymorphism described previously.[5] (Lane *3*) 445-bp PCR fragment (primer combination a): 5-repeat homozygote. (Lane *4*) 397- and 301-bp PCR fragment (primer combination a): 2-, 4-repeat heterozygote). (Lane *5*) 247-bp PCR fragment (primer combination b): 2-repeat homozygote.

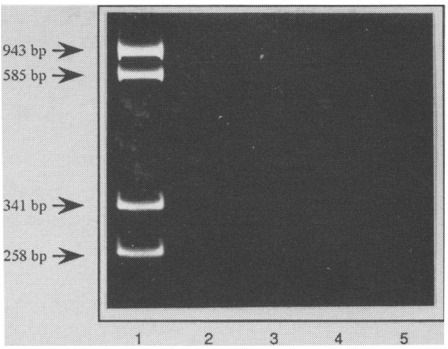

FIGURE 2 c^7dGTP containing PCR fragments of the DRD4 gene stained with ethidium bromide. Aliquots of 13 μl of the identical PCR reaction mixtures used for Fig. 1 were electrophoresed and stained with ethidium bromide. (Lane *1*) 0.5 μg molecular weight marker pUC18 digested with *Sau*3A (sizes at *left*). (Lane *2*) 477-bp PCR fragment (primer combination c). (Lanes *3–5*) The DRD4 polymorphism described previously.[5] Lane *3*) 445-bp PCR fragment (primer combination a): 5-repeat homozygote. (Lane *4*) 397- and 301-bp PCR fragment (primer combination a): 2-, 4-repeat heterozygote. (Lane *5*) 247-bp PCR fragment (primer combination b): 2-repeat homozygote.

RESULTS AND DISCUSSION

The application of c^7dGTP instead of dGTP proved to be a very useful method for amplification of genomic DNA fragments with very high G/C content. Because it is virtually impossible to stain c^7dGTP containing DNA with ethidium bromide, we looked for an easy, quick, and low-priced alternative.

Investigators usually try to avoid the staining problem by radioactive labeling of PCR primers or using ^{32}P-labled nucleotides. These methods can be disagreeable, are more expensive, and are much more time-consuming than the application of c^7dGTP. Besides, amplification reactions with radiolabeled oligonucleotides often yield high background, especially if the amount of amplification product is low or only moderately high and needs some time to adjust all parameters to optimal conditions.

We tested silver staining as a method for visualizing c^7dGTP containing DNA fragments. The binding of silver ions is obviously not impaired by c^7dGTP. Figures 1 and 2 illustrate the striking difference between c^7dGTP-containing PCR fragments stained with ethidium bromide and silver nitrate, respectively. As well as being more efficient and less costly, application of c^7dGTP is more sensitive than ethidium bromide staining.

ACKNOWLEDGMENTS

We thank Dr. Hans-Jürgen Mägert for critical reading of this manuscript and Wolfgang Posselt for his help with photography.

REFERENCES

1. McConlogue, L., M.A.D. Brow, and M.A. Innis. 1988. Structure-independent DNA amplification by PCR using 7-deaza-2′-deoxyguanosine. *Nucleic Acids Res.* **16 (20):** 9869.
2. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Ehrlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–494.
3. Seela, F. and H.-J. Driller. 1989. Alternating $d(G\text{-}C)_3$ and $d(C\text{-}G)_3$ hexanucleotides containing 7-deaza-2′-deoxyguanosine or 8-aza-7-deaza 2′-deoxyguanosine in place of dG. *Nucleic Acids Res.* **17(3):** 901–910.
4. Seela, F. and A. Röling. 1991. 7-Deazapurine containing DNA: Efficiency of c^7G_dTP, c^7A_dTP and c^7I_dTP incorporation during PCR-amplification and protection from endodeoxyribonuclease hydrolysis. *Nucleic Acids Res.* **20(1):** 55–61.
5. Van Tol, H.H.M., C.M. Wu, H.C. Guan, K. Ohara, J.R. Bunzow, O. Civelli, J. Kennedy, P. Seeman, H.B. Niznik, and V. Jovanovic. 1992. Multiple dopamine D4 receptor variants in the human population. *Nature* **358:** 149–152.

Received June 21, 1994; accepted in revised form August 19, 1994.