Synthesis *in vitro* and application of biotinylated DNA probes for human papilloma virus type 16 by utilizing the polymerase chain reaction

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The polymerase chain reaction (PCR) has been used to incorporate biotinylated deoxynucleotide triphosphate analogues into a 120 bp sequence from the E6 region of human papilloma virus type 16 (HPV16). No loss of amplification efficiency is observed utilizing concentrations of up to 200 μM -biotin-11-dUTP, or 180 μM -biotin-7-dATP and -biotin-16-dUTP (where the numbers refer to the number of carbon atoms in the spacer arms). Internally biotinylated PCR products can be detected following slot-blot or vacuum transfer to nitrocellulose or nylon filters without prior electrophoretic separation of the reactants, since unincorporated biotinylated analogues pass through the filter. Internally biotinylated PCR products can also be applied as hybridization probes in Southern blot analysis or *in situ* hybridization. This system enables detection of PCR products or target sequences at levels below that for 5'-biotinylated probes and can be applied in an 'open sandwich assay' without the need for a separate labelled probe currently required in conventional sandwich assays. However, as hybridization probes, sensitivity may be limited by the steric hindrance of strand hybridization possibly due to the spacer arms linking the nucleotides to the biotin molecule.

INTRODUCTION

The problems inherent in the use of radioactive oligonucleotide probes for the detection of complementary nucleic acid sequences by specific hybridization have, in recent years, generated considerable interest in the use of non-radioactive labelling systems (reviewed in [1]). Non-radioactive labelling systems may lead to significantly improved detection limits and the use of oligonucleotides linked to enzymes has been demonstrated to produce sensitivities comparable to those of ^{32}P -labelled probes [2–9].

Techniques for the biotinylation of oligonucleotides by direct synthesis, nick translation [10], photobiotinylation [11] and incorporation of biotinylated deoxynucleotide analogues [12] have been described. Biotin labelling eliminates many of the problems associated with the use of radioactivity and permits the use of a wide range of endpoint detection and signal amplification systems [1]. Biotinylated probes have been applied to the detection of target DNA and RNA sequences in a variety of techniques such as Southern and Northern blotting, *in situ* hybridization and sandwich assays.

We have investigated the use of the polymerase chain reaction (PCR) for the synthesis *in vitro* of biotin-labelled DNA probes, their use in the detection of target sequences and for the detection of amplified sequences. The model used is that for the amplification of a 120 bp sequence from the E6 region of human papilloma virus type 16. The human papilloma viruses (HPVs) are a family of double-stranded-DNA viruses associated with both benign and malignant disease. HPV16 is associated with cervical carcinoma [13–17] but has also been found in normal tissue [18–21]. Utilizing several biotinylated deoxynucleotide triphosphate analogues we have demonstrated that sequences internally labelled in the PCR may have applications and advantages in hybridization assays and for the direct detection of PCR products without prior gel electrophoresis.

MATERIALS AND METHODS

Synthesis of oligonucleotides

Oligonucleotide synthesis was performed on a Biotech Instruments Ltd. BT8500 DNA synthesizer employing cyanoethyl phosphoramidites by Dr J. Fox (Alta Bioscience). The protecting 3' group was removed and oligonucleotides used directly following reverse-phase h.p.l.c. or isolation from a 20 % acrylamide/8 M-urea gel preceding butan-1-ol extraction. Oligonucleotides were adjusted to 1 pmol/ml and stored at -20°C . Oligonucleotide primers complementary to the sequence in the E6 region of HPV16 DNA were designed as previously described [22] to generate a 120 bp fragment following amplification in the PCR. Another oligonucleotide was synthesized complementary to a mid-region sequence within the E6 region of HPV16 DNA. Forward and reverse oligonucleotide primers were also synthesized with a 5'-biotin molecule using Aminolink 2 (Applied Biosystems Ltd) methodology.

Amplification of DNA

Amplification of the target HPV16 DNA isolated from CaSki cells, a cervical carcinoma line carrying high copy numbers of the HPV16 genome, was achieved using the PCR as described by Saiki *et al.* [23]. CaSki DNA (20 pg) and 1 μM final concentration of primers 6139 (TCAAAGCCACTGTGTCCTG) and 5198 (CGTGTTGATGATCTGCA) were placed in 100 μl of a reaction mixture containing 67 mM-Tris/HCl (pH 8.8), 16.6 mM- $(\text{NH}_4)_2\text{SO}_4$, 6.7 mM-MgCl₂, 10 mM-2-mercaptoethanol, 6.7 mM-EDTA, 170 μg of BSA/ml and 200 μM of each dNTP (dATP, dCTP, dTTP, dGTP; BRL). The reaction mix was heated to 98°C for 10 min, 4 units of *Taq* DNA polymerase from *Thermus aquaticus* (Anglian Biotech Ltd.) was added and the mix was overlaid with 50 μl of heavy mineral oil (Sigma). The reaction mix was taken through up to 30 cycles of amplification in a Wolfson Research Laboratories programmed thermal cyclor.

Abbreviations used; PCR, polymerase chain reaction; HPV16, human papilloma virus type 16; BSA, bovine serum albumin; HRP, horse radish peroxidase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; bio, biotin.

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Each cycle comprised 0.5 min at 94 °C, 0.5 min at 40 °C, followed by 1.5 min at 72 °C.

Incorporation of biotin into the polymerase chain reaction product

Three methods of biotin incorporation were used:

(a) 5'-Biotinylated primers were substituted for all the unmodified primers in the reaction mix.

(b) Biotin-11-deoxyuridine (bio-11-dUTP), biotin-16-deoxyuridine (bio-16-dUTP) or biotin-7-deoxyadenosine (bio-7-dATP) (BCL) (the numbers refer to the numbers of carbon atoms in the spacer arms) were substituted for the appropriate unlabelled dNTP in the reaction mix before preheating. The ratio of biotinylated to unlabelled dNTP was varied (see below) while maintaining a total of 200 μ M of each base.

(c) Biotinylated dNTP (100 μ M) was added to the standard reaction mixture before the penultimate cycle of amplification.

Detection of amplified DNA

After the last amplification cycle, 20 μ l samples were electrophoresed on a 1.5% agarose gel at 5 V/cm, denatured in alkaline solution and transferred to Hybond nylon membrane (Amersham International) by vacuum blotting (Hybaid) for 40 min. Filters were incubated in 3% BSA in PBS for 3 h followed by incubation with streptavidin-horseradish peroxidase complex (streptavidin-HRP; Amersham International) at a 1:300 dilution in PBS for 30 min. Following three 10 min washes in PBS, filters were stained in 100 ml of 0.1 M-Tris/HCl (pH 7.6) containing 50 mg of diaminobenzidine (Sigma) and 1 ml of 1% H₂O₂. A brown precipitate formed on the filter and after 5 min the reaction was stopped by washing in water and the filter was air-dried.

Filters were also probed with a mid-sequence HPV16 specific 40-mer oligonucleotide (6815:AAAGATTCCATAATATAAG-GGGTCGGTGGACCGGTGCGATG) which had been hexanucleotide-labelled with ³²P. After washing, the filters were exposed to X-ray film for 24–48 h at –80 °C. This procedure was performed before and after staining the filter with streptavidin-HRP and diaminobenzidine.

Slot-blotting

Following amplification, 0.1–20 μ l samples of PCR reactants with 100 μ M-bio-7-dATP but with and without CaSki DNA were applied to nylon membranes using a BRL slot-blot manifold. Transfer occurred in 1 $\times$ SSC (SSC is 0.15 M-NaCl/15 mM-sodium citrate) buffer, and the membranes were u.v.-irradiated for 5 min and baked at 80 °C for 30 min prior to diaminobenzidine staining as described above.

Hybridization *in situ*

Slides of cell lines (CaSki, HeLa, Raji) were prepared by cytocentrifugation onto acid-washed glass slides, fixed at –20 °C in methanol/acetic acid (3:1, v/v) for 10 min, air-dried and subsequently stored at –20 °C. Before hybridization, cells were incubated for 30 min in methanol containing 2% H₂O₂ followed by 10 min in ethanol prior to air drying; then 10 μ l of hybridization cocktail consisting of 50% deionized formamide, 10% (w/v) dextran sulphate, 2 $\times$ SSC, 1 mg of denatured calf thymus DNA/ml and biotinylated DNA probe was applied to each slide and covered with a rubber cement-sealed sterile glass coverslip. The tissue section and cells were denatured at 85 °C for 10 min before hybridization at 37 °C overnight. Coverslips were removed and slides sequentially washed in 2 $\times$ SSC (5 min at room temperature), 0.1 $\times$ SSC (10 min at 45 °C), 2 $\times$ SSC (5 min at room temperature), 0.1% Triton X-100 in PBS (5 min at room

temperature) and finally rinsed in PBS. The hybridized biotinylated probe was then detected using immunocytochemical techniques. Briefly, slides were incubated sequentially at 37 °C in mouse anti-biotin monoclonal antibody (Dako Ltd.) for 60 min, in biotinylated rabbit anti-(mouse IgG) (Dako Ltd.) for 30 min and in streptavidin-biotinylated-HRP complex (Dako Ltd.) for 60 min. Intervening washes were made in PBS for 10 min and all antibody dilutions were made in PBS containing 10% normal goat serum. After the final incubation step, the slides were washed at room temperature in PBS for 10 min, treated with 0.05 M/HCl (pH 7.6)/0.15 M-NaCl (TBS) containing 0.6 μ g of diaminobenzidine/ml and 0.03% H₂O₂ for around 5 min and washed in PBS. If necessary, gold/silver amplification of the diaminobenzidine signal was made by the method of Burns *et al.* [24] using a diaminobenzidine enhancement kit (Amersham International). Finally, tissues and cells were counter-stained with Toluidine Blue, washed in tap water, dehydrated, air dried and mounted in DPX.

RESULTS

We have investigated the incorporation of bio-11-dUTP, bio-16-dUTP and/or bio-7-dATP into the PCR product formed using CaSki DNA and HPV16 specific primers using different concentrations of the biotinylated dNTPs in the reaction mix. We can demonstrate several advantages in employing the PCR to incorporate biotinylated analogues of dATP and dTTP, suggesting that this technique is ideally suited as a routine labelling system for generation of DNA probes, although with some caveats.

Fig. 1(a) shows a 1.5% agarose gel containing 500 ng of ethidium bromide/ml under u.v. illumination, showing the separation of 15 μ l samples of PCR products generated using 1.5, 3, 12, 25, 50, 100 or 200 μ M-bio-11-dUTP and 20 pg of CaSki DNA. Thirty amplification cycles of the PCR produced a single band corresponding to a fragment of approx. 123 bp using no bio-11-dUTP to 100 μ M-bio-11-dUTP. Totally replacing dTTP with 200 μ M-bio-11-dUTP however produced a single band corresponding to a fragment with significantly reduced electrophoretic mobility corresponding to an apparent size of approx. 246 bp. The incorporation of bio-11-dUTP into the HPV16 amplified sequence was demonstrated following transfer of the bands to a nylon membrane filter. Staining with streptavidin-HRP followed by the substrate diaminobenzidine reveals positive correlation between the amount of staining and the initial concentration of bio-11-dUTP in the reaction mixes. The highest signal was achieved using 200 μ M-bio-11-dUTP (instead of 200 μ M-dTTP), with no visible staining of the band generated using 1.5 μ M-bio-11-dUTP (Fig. 1b). Identical results were obtained if varying concentrations of bio-7-dATP or bio-16-dUTP (results not shown) were substituted for the equivalent amount of dATP or dTTP respectively, except that no amplification was evident if dATP or dTTP was totally replaced by 200 μ M of the biotinylated analogue. The absence of amplification obtained using 200 μ M-bio-7-dATP was confirmed by diaminobenzidine staining of vacuum-transferred bands onto a nylon filter. No diaminobenzidine precipitate was visible for the band generated using 1.5 μ M- or 3 μ M-bio-7-dATP or bio-16-dUTP. Inclusion of 100 μ M-bio-11-dUTP and 100 μ M-bio-7-dATP either singly or in combination in the initial PCR reaction mixture did however result in decreased efficiency of amplification as determined by ethidium bromide staining, but the intensity of staining with diaminobenzidine was enhanced. Slot blotting of PCR reactants generated with 100 μ M-bio-7-dATP revealed that using samples of greater than 0.5 μ l gave positive staining with diaminobenzidine (Fig. 2). Reaction samples generated in the

Fig. 1. Analysis of PCR products in presence of bio-11-dUTP
(a) 1.5% agarose gel in ethidium bromide (500 ng/ml) showing separation of 15 µl samples of PCR reactions performed using primers 6139 and 5198 and CaSki DNA. Thirty amplification cycles were performed in the presence of 1.5, 3, 12, 25, 50, 100, or 200 µM-bio-11-dUTP. Positions of the 123, 246 and 369 bp markers are indicated. (b) Bands from (a) transferred to nylon membrane (Hybond) and stained with streptavidin-HRP followed by diaminobenzidine. (c) Bands from (b) probed with ³²P-labelled oligonucleotide 6815 specific for mid region of HPV16 PCR product.

presence of 100 µM-bio-7-dATP but lacking CaSki template gave no signal following diaminobenzidine staining.

Since bio-11-dUTP from 3–200 µM or bio-7-dATP from 3–180 µM in the reaction mix produced detectable PCR amplified sequences after 30 amplification cycles, we investigated the number of amplification cycles that would be required for detection by diaminobenzidine staining of the biotinylated amplified product using different initial concentrations of the biotinylated dNTP (Table 1). It can be seen that fewer amplification cycles (and hence overall reaction times) are required compared to the number of cycles required for detection of PCR products generated using 5'-biotinylated primers. Inclusion of 100 µM-bio-7-dATP to the reaction mix during the penultimate amplification cycle resulted in detection only if added after 19, 24 or 29 cycles. The intensity of staining of these products was also considerably less (< 50% by densitometric scanning) than that obtained by addition of 100 µM-bio-7-dATP before the initial cycle.

The effect of incorporating biotinylated dNTPs into the amplified HPV16 DNA sequence in the PCR on its ability to

Fig. 2. Diaminobenzidine staining of slot-blotted HPV16 PCR reactants after 30 cycles using 100 µM-bio-7-dATP in the presence (+) or absence (-) of CaSki DNA template
Volume of samples applied: 1, 0.1 µl; 2, 0.5 µl; 3, 1.0 µl; 4, 5.0 µl; 5, 10 µl; 6, 20 µl.

Table 1. Minimum numbers of PCR cycles required for diaminobenzidine detection of amplification products generated using different biotinylation strategies

Strategy	Number of PCR cycles
(a) 5'Biotinylated primers	30
(b) 100 µM-bio-7-dATP added during penultimate cycle	20
(c) Addition of bio-11-dUTP at cycle 0 at:	
50 µM	15
100 µM	15
200 µM	12

hybridize to complementary DNA sequences was investigated in Southern blotting and *in situ* hybridization. Fig. 1(c) shows an autoradiograph produced following the hybridization of the ³²P labelled 40-mer oligonucleotide 6815 (specific for a mid-region sequence of the HPV16 amplified DNA) to the PCR product generated using varying concentrations of bio-11-dUTP and 30 amplification cycles followed by staining with streptavidin-HRP and diaminobenzidine. The ³²P-labelled probe bound to amplified sequences produced using 1.5–100 µM-bio-11-dUTP but not when 200 µM-bio-11-dUTP was used in the reaction mix. The intensity of the signal is reduced as the amount of bio-11-dUTP is increased in the reaction mix and incorporated into the product. The hybridization of the ³²P-labelled probe to the biotinylated amplified sequence before staining with streptavidin-HRP and diaminobenzidine was similar except that the PCR product generated using 200 µM-bio-11-dUTP was weakly detected (results not shown). Hybridization of biotinylated ³²P-labelled PCR products to PCR-amplified (with or without biotin incorporation) or CaSki HPV16 DNA on a Southern blot is demonstrated in Fig. 3. The use of the biotinylated PCR product generated using 100 µM-bio-11-dUTP in the reaction mix in *in situ* hybridization to detect HPV16 in CaSki cells is shown in Fig. 4.

Fig. 3. Comparison of sensitivities obtained when probing HPV16 CaSki DNA (lanes 2–4) or PCR-amplified HPV16 generated with (lane 6) or without (lane 5) 100 μ M-bio-11-dUTP

Samples were vacuum-transferred from gel (a) to nylon, probed with HPV16 PCR product generated using 100 μ M-bio-11-dUTP and stained with diaminobenzidine (b). Filter (b) was then probed with 32 P-labelled HPV16 PCR product and autoradiographed for 24 h (c).

Fig. 4. Detection of HPV16 by *in situ* hybridization

(a) CaSki cells containing HPV16 probed by *in situ* hybridization using the HPV16 PCR product generated in the presence of 100 μ M-bio-11-dUTP. Arrow indicates staining of HPV16 sequences around nuclei following streptavidin–biotinylated horseradish peroxidase complex incubation and diaminobenzidine enhancement. Slides were counter-stained with Toluidine Blue. (b) HPV16-negative HeLa cells treated as in (a).

DISCUSSION

The polymerase chain reaction is readily amenable to the incorporation of deoxynucleotide triphosphate analogues into the amplified DNA sequence. It has been demonstrated that 5-

bromo-2'-deoxyuridine-5'-triphosphate [25], [α - 32 P]CTP, 7-deaza-2'-deoxyguanosine and inosine triphosphate nucleotides may be readily incorporated, the latter finding application in resolution of problematic reading regions of DNA sequences. The incorporation of biotinylated dNTPs into PCR amplified sequences has been demonstrated [12] using 50 μ M-bio-11-dUTP in the reaction mix. This internally biotinylated PCR product was also demonstrated to be applicable as a probe in Southern blot analysis.

We have investigated the incorporation of bio-11-dUTP, bio-16-dUTP and bio-7-dUTP into the PCR amplified products and the applicability of the PCR products as probes. The replacement of dTTP with 1.5–200 μ M-bio-11-dUTP in the initial reaction mix did not prevent primer extensions in the HPV16 system described (Fig. 1). However, concentrations above 180 μ M of bio-16-dUTP or bio-7-dATP prevented primer extension. The length of the spacer arm in terms of carbon atoms is therefore critical in determining the efficiency of primer extension or annealing, presumably due to steric hindrance effects. Increased steric hindrance is expected for the bio-7-dATP which contains a C_7 spacer arm, but it is surprising that a similar effect is evident using a C_{16} spacer arm. These data suggest that a C_{11} spacer arm is possibly optimal.

Internally biotinylated PCR products enabled the detection of PCR amplified HPV16 sequences in Southern blots (see Fig. 3) and in *in situ* hybridization (Fig. 4). However, internally biotinylated PCR products generated using > 100 μ M of biotinylated analogues in the reaction mix reduced the availability of the sequence to hybridize with its complementary unmodified sequence and hence the detection sensitivity achieved. There would appear to be steric hindrance of the hybridization or increased dissociation of the double strand as the amount of incorporated biotinylated dNTP increases. It is possible that the hybridization efficiency may be improved by the use of less stringent washes, but this may be at the expense of specificity. There is clearly a trade off point between sensitivity of detection and specificity. The incorporation of other forms of biotinylated deoxynucleotide analogues with different length spacer arms and biotin attachment sites may overcome these problems. With or without signal amplification, we have been unable to achieve detection sensitivities comparable with those obtained with radionuclide-labelled probes (see Fig. 3).

The incorporation of biotinylated dNTPs in the PCR can however be seen to offer several advantages. High levels of incorporation can be achieved, enabling more sensitive detection than achieved using 5'-biotinylated probes or PCR products.

Fig. 5. Schematic representation of (a) conventional sandwich assay, and (b) 'open-sandwich' assay for specific detection of PCR products

This enables the PCR to be used for the production of specific DNA sequences for probes and may obviate the need for signal amplification systems. It is however possible to use signal amplification systems in those situations where increased sensitivity is desirable. Many endpoint systems are applicable, based on the specificity and affinity of streptavidin for biotin. This labelling system also has application in sandwich assays used for detecting PCR amplification products which currently require 5'-biotinylated probes and signal amplification systems for detection. Utilizing the system outlined in this report, it is possible to envisage an 'open-sandwich' assay whereby biotinylated PCR products hybridize to an immobilized oligonucleotide (see Fig. 5) and are detected by incubation of the complex with streptavidin linked to a signal-generating molecule. It is also possible to detect PCR amplification products after fewer amplification cycles than when using 5'-biotinylated primers (or addition of biotinylated analogues during penultimate amplification cycle), although ethidium bromide staining of electrophoresed reactants has an equivalent sensitivity. However, since unincorporated biotinylated dNTPs can be demonstrated to pass through nylon filters upon vacuum blotting/dot blotting whereas biotinylated dNTPs incorporated into the PCR product remain on the filter, it is possible to detect specifically PCR amplification products on filters without electrophoretic separation of the reactants (Fig. 2). This is only possible provided that the PCR system is

optimized, as demonstrated by the HPV16 system, for the amplification of the target sequence defined by the primers with no amplification of non-target sequences.

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REFERENCES

- Matthews, J. A. & Kricka, L. J. (1988) *Anal. Biochem.* **169**, 1-25
- Urdea, M. S., Warner, D., Running, J. A., Stempien, M., Clyne, J. & Horn, T. (1988) *Nucleic Acids Res.* **16**, 4937-4956
- Richardson, R. W. & Grumport, R. I. (1983) *Nucleic Acids Res.* **11**, 6167-6184
- Li, P., Medon, P. P., Skingle, D. C., Lanser, J. A. & Symons, R. H. (1987) *Nucleic Acids Res.* **15**, 5275-5287
- Draper, D. E. & Gold, L. (1980) *Biochemistry* **19**, 1774-1781
- Syvanen, A. C., Alanen, M. & Soderlund, H. (1985) *Nucleic Acids Res.* **13**, 2789-2802
- Renz, M. & Kurz, C. (1984) *Nucleic Acids Res.* **12**, 3435-3444
- Woodhead, J. L. & Malcolm, A. D. B. (1984) *Biochem. Soc. Trans.* **12**, 279
- Jablonski, E., Moomaw, E. W. & Ruth, J. L. (1986) *Nucleic Acids Res.* **14**, 6115-6128
- Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237-251
- Forster, C. A., McInnes, J. L., Skingle, D. C. & Symons, R. H. (1985) *Nucleic Acids Res.* **13**, 745-755
- Lo, Y. M. D., Mehal, W. Z. & Fleming, K. A. (1988) *Nucleic Acids Res.* **16**, 8719
- Walker, P. G., Singer, A., Dyson, J. L., Shah, K. V., To, A. & Colman, D. V. (1983) *Br. J. Cancer* **48**, 99-101
- Boshart, M., Gissman, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W. & Zur Hausen, H. (1984) *EMBO J.* **3**, 1151-1157
- Beaudenon, S., Kremsdorf, D., Croissant, O., Jablonska, S., Wain-Hobson, S. & Orth, G. A. (1986) *Nature (London)* **321**, 246-249
- Lorincz, A. T., Quinn, A. P., Lancaster, W. D. & Temple, G. F. (1987) *Virology* **159**, 187-190
- McCane, D. J. (1986) *Biochim. Biophys. Acta* **283**, 205-209
- Toon, P. G., Arrand, J. R., Wilson, L. P. & Sharp, D. S. (1986) *Br. Med. J.* **293**, 1261-1264
- Cox, M. F., Meanwell, C. A., Maitland, N. J., Scully, C., Blackledge, G. & Jordan, J. A. (1986) *Lancet* **ii**, 157-158
- Gergely, L., Czegledy, J. & Hernady, Z. (1987) *Lancet* **ii**, 513
- De Villiers, E. M., Wagner, D., Schneider, A., *et al.* (1987) *Lancet* **ii**, 703-705
- Young, L. S., Bevan, I. S., Johnson, M. A., *et al.* (1989) *Br. Med. J.* **298**, 14-18
- Saiki, R. R., Gelfand, D. H., Stoffel, S., *et al.* (1988) *Science* **239**, 487-491
- Burns, J., Chan, V. T. W., Jonasson, J. A., Fleming, K. A., Taylor, S. & McGee, J. O'D. (1985) *J. Clin. Pathol.* **38**, 1085-1092
- Niedobitek, G., Finn, T., Herbst, H., Bornhofs, G., Gerdes, J. & Stein, H. (1988) *Am. J. Pathol.* **131**, 1-4

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