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Inversion of *in situ* synthesized oligonucleotides: improved reagents for hybridization and primer extension in DNA microarrays

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ABSTRACT

Oligonucleotides synthesized in array format suffer from contamination by truncated species. We have developed a method to invert DNA molecules *in situ* after completed synthesis. Reactive functions at the 5′-ends of the oligonucleotides are permitted to react with functions on the support before the 3′-ends are released, in effect reversing the orientation of full-length oligonucleotides, while any 5′-truncated molecules are lost. This strategy serves both to purify *in situ* synthesized reagents and to reorient the oligonucleotides, causing them to expose free 3′-hydroxyls. *In situ* inverted oligonucleotides can be used in assays based on DNA polymerase-assisted extension of immobilized primers, and we demonstrate their utility in minisequencing and in pyrosequencing.

INTRODUCTION

Oligonucleotide arrays, i.e. sets of oligonucleotides distributed in a fixed pattern on the surface of a planar device, can be used for simultaneous analysis of many genetic factors in a sample (1). Two distinct approaches are principally used to construct oligonucleotide arrays. Individual oligonucleotides may be manufactured separately and purified before they are immobilized in defined patches on a planar solid phase (2). This allows good control over the quality of the immobilized reagents, but arrays of higher complexity are increasingly more difficult to manufacture.

In the other major approach to production of arrays, sets of oligonucleotides are built stepwise directly on a surface (3,4). Typically, this chemical DNA synthesis proceeds in a 3′→5′ direction. By applying optical masking during light-directed deprotection, arrays of very high complexity can be made. The arrays can be manufactured at a limited cost and are in use experimentally to distinguish or quantitate large sets of nucleic acid target sequences. Because the reagents are constructed *in situ*, it has not been possible to ensure that all oligonucleotide molecules are of the desired composition. In practice, oligonucleotide arrays constructed in this fashion are heavily contaminated with truncated molecules (5). Urdea and Horn have suggested using exonucleases to remove any support-bound oligonucleotides lacking a 5′-terminal blocking function, as a means to purify solid phase synthesized oligonucleotides *in situ*. However, their method was unable to hydrolyze fragments shorter then 10 bases when CPG was used as the solid phase (6). We have been unable to remove failure sequences by exonuclease digestion using the polystyrene supports that we used herein (M.Nilsson, unpublished results).

Most commonly, oligonucleotides immobilized in arrays are employed to interrogate nucleic acid samples by relying on the differential hybridization stability of target molecules that are perfectly base-paired to an immobilized probe versus ones that are mismatched at one or more nucleotide positions. Truncated probes, interacting less stably with the target sequences, may contribute to a poor signal-to-noise ratio.

Increasingly, DNA polymerases are applied to enhance the distinction of target sequence variants on DNA arrays, by determining which nucleotide is incorporated at the 3′-end of a primer that hybridizes just upstream of a variable nucleotide position in the target. This method is known as minisequencing or single nucleotide primer extension (7). Minisequencing on arrays requires that the oligonucleotides present free 3′-hydroxyls, but *in situ* synthesis in the 5′→3′ direction has an even greater tendency to yield truncated molecules than synthesis in the conventional 3′→5′ direction.

Synthesis of oligodeoxynucleotides 3′→5′ involves nucleophilic attack of a primary 5′-hydroxyl in the growing oligonucleotide chain on a trivalent phosphorus atom at the 3′-position of an incoming phosphoramidite. In contrast, 5′→3′ synthesis requires a secondary 3′-hydroxyl to attack a phosphorus atom located at the 5′-position of a phosphoramidite. Secondary hydroxyl groups are less efficient nucleophiles than primary hydroxyls. With a single exception (8), it is a common observation that chemical synthesis in the 5′→3′ direction proceeds with a lower yield compared to the usual direction of synthesis (9–12). While 3′-truncated probes may compromise hybridization stability, the presence of such molecules are of even greater concern in primer extension assays, where truncated molecules of different length would interrogate different nucleotide positions.

We now report a technique in which the orientation of oligonucleotides synthesized in the conventional 3′→5′ direction is reversed *in situ*, resulting in immobilized oligonucleotides that

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expose free 3′-hydroxyls. The inversion procedure removes any truncated molecules, generating a homogeneous population of full-length oligonucleotides, suitable for both hybridization- and polymerase-mediated analyses of target nucleic acid sequences.

MATERIALS AND METHODS

Reagents

Anhydrous pyridine and dichloromethane was obtained from Aldrich. Triethylamine was dried and distilled before use. Phosphoramidite **3** (Fig. 1) was prepared by condensation of 5′-*O*-DMTr-thymidine-3′-*O*-succinate with 5-amino-1-pentanol and phosphitylation of the obtained hydroxyl derivative according to the standard procedure (13).

Oligonucleotide inversion

Cassettes charged with aminometyl polystyrene (22 μmol/g, 10 mg, 0.2 μmol) (a kind gift from Dr Andrus, PE Applied Biosystems) were placed in the oligonucleotide synthesizer (Applied Biosystems 394 DNA Synthesizer) and subjected to three consecutive couplings with triethyleneglycol phosphoramidite **1** (Figs 1 and 2). The standard acetic anhydride/1-methylimidazole capping procedure was used throughout all oligonucleotide syntheses. Next, mixtures of **1** and levulinyl-protected phosphoramidite **2** were used for coupling to the support. The resulting partially capped supports were subsequently extended by coupling the cleavable phosphoramidite **3**, followed by synthesis of a mixed oligonucleotide sequence. The final 5′-*O*-DMTr protecting groups were removed and solid phase-bound oligonucleotides were phosphorylated using a 0.1 M *o*-chlorophenylphosphorobistriazolide solution in pyridine:acetonitrile 1:1 for 10 min. The obtained phosphodiester monotriazolide was hydrolyzed with freshly prepared triethylammonium bicarbonate (0.3 M, pH 7.5) for 5 min. Thereafter supports were washed with acetonitrile and subjected to a mixture of 0.1 M hydrazine in pyridine:acetic acid 4:1 for 10 min to remove the levulinyl function. Solid phases were transferred to separate Eppendorf tubes, washed with dry pyridine (2 × 1 ml), and treated with 0.1 M mesitylene sulfonyl-3-nitro-1,2,4-triazole (MSNT) in pyridine for 2 h with occasional shaking. Following this condensation, supports were washed with acetonitrile (3 × 1 ml), and treated with a mixture of 4-nitrophenylbenzaldoxime and tetramethylguanidine in dioxane:water 1:1 for 16 h. Reaction mixtures were transferred to larger flasks with tight screw caps, and incubated at 60°C for 16 h after addition of concentrated aqueous ammonia (2 ml). Next, the mixtures were transferred to round-bottom flasks and all volatile matter was evaporated *in vacuo*. The residues were treated with 80% aqueous acetic acid for 2 h to cleave inverted material from the support. Finally, mixtures were evaporated, co-evaporated twice with water (2 ml), and analyzed by capillary electrophoresis on a Beckman MDQ system using a ssDNA 100-R capillary kit (capillary length 30 or 50 cm).

To demonstrate the purity of the inverted oligonucleotides, the ammoniacal fraction was separated from the support. The solid phase was washed with water and treated with acetic acid. Both phases were analyzed separately.
(ABI), in order to obtain sufficient products to monitor the

Primer extension from support-bound oligonucleotides

Minisequencing. A 19mer oligodeoxynucleotide 5′-GGCT-GAGTATGTGGTCTAT-3′ was synthesized at the 0.2 μmol scale on polystyrene beads treated with phosphoramidites **1** and **2** in a 1:1 ratio, as described. This material was inverted and left on the support.

The complementary template strand was a synthetic oligonucleotide with the sequence 5′-GGCTGAGTATGTGGTC-TATGTCGTCGTTCG-3′. An aliquot of 0.2 mg of support was used in each of four separate primer extension reactions, containing different ^{3}H-labeled deoxynucleotide triphosphates. The 50 μl reactions contained 50 mM Tris–HCl, pH 8.8, 15 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 0.01% (w/v) gelatin, 10 μM [^{3}H]dNTP (0.1 μCi) (Amersham), 0.2 μM template, and 5 U *Taq* DNA polymerase (AmpliTaq; Perkin Elmer). The reactions were incubated at 50°C for 10 min. Beads were washed and centrifuged twice with 200 μl buffer (40 mM Tris–HCl pH 8.8, 1 mM EDTA, 50 mM NaCl, 0.1% v/v Tween-20, 10% v/v ethanol), and then placed in scintillation liquid (Optiphase Supermix; Wallac, Finland) and counted in a β counter (Wallac 1414).

Pyrosequencing. Pyrosequencing experiments were performed using the above solid phase and with the same template as for the minisequencing reaction. Sequential nucleotide triphosphate additions were performed as described (14), with detection of the liberated pyrophosphate as a byproduct of dNTP incorporation. The enzymatic luminometric pyrophosphate detection assay used to detect the released pyrophosphate contained a mixture of the enzymes DNA polymerase Klenow fragment, ATP sulfurylase, luciferase and apyrase (15). Nucleotides were added at 80 s intervals to allow degradation of unincorporated dNTPs by apyrase before the next addition. The reactions were performed on a prototype apparatus from Pyrosequencing AB (Uppsala, Sweden).

RESULTS AND DISCUSSION

In search of a method to synthesize oligonucleotides in DNA microarrays having free 3′-ends, we became attracted by the idea of synthesizing oligonucleotides in the standard 3′→5′ direction and then reversing their orientation *in situ*.

This requires a function at the 5′-end of the oligonucleotides capable of reacting with appropriate functions on the support before the 3′-ends are released. The positioning of oligonucleotides in the array would be preserved, since in this approach the molecules remain bound to the support at all times through at least one end.

We have chosen a strategy in which the 5′-ends may react with functions on the support, while the rest of the molecule remains fully protected. The secondary solid phase attachment of the oligonucleotides takes place in a water-free solvent. This offers a wide selection of reactive functionalities, and the risk of side-reactions is minimized.

Oligonucleotide inversion

Synthesis on planar solid supports results in a limited quantity of product. Therefore, all model oligonucleotide syntheses were performed on 50–70 μm diameter polystyrene beads individual steps of the inversion procedure.

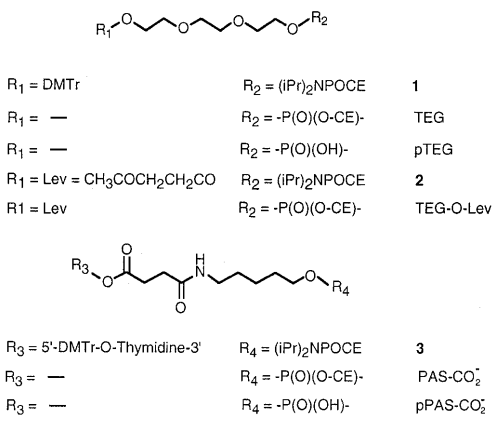

Figure 1. Compounds used in the study.

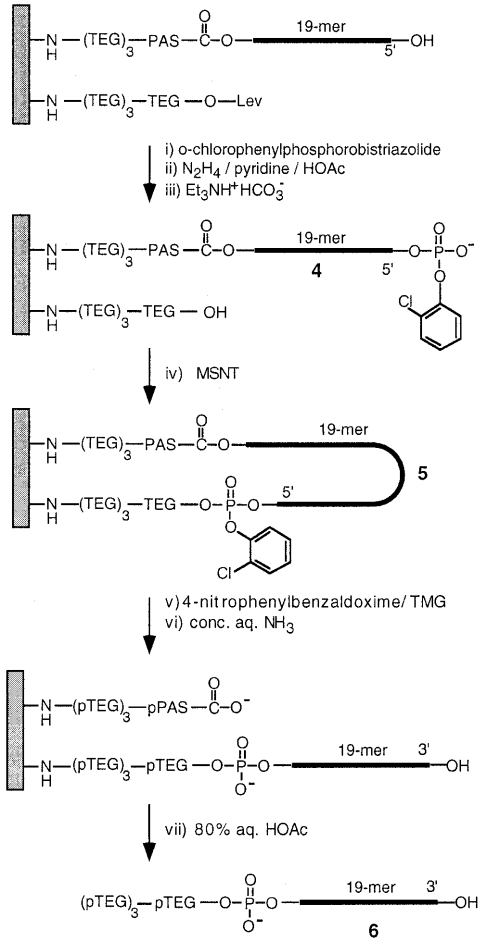

Figure 2. *In situ* inversion of oligonucleotides synthesized on a solid support.

A triethyleneglycol phosphoramidite **1** (Fig. 1) was coupled to the methylamino-derivatized polystyrene beads, resulting in the formation of a phosphoramide linkage (Fig. 2). This linkage is stable under conditions of oligonucleotide synthesis, inversion and ammoniacal deprotection, but if desired it can be cleaved by aqueous acetic acid (16). Thereby, all reaction products can be characterized by capillary electrophoresis.

The synthesis was continued by coupling two additional triethyleneglycol residues as a linker. This was done to ensure that inverted and non-inverted material could be distinguished by capillary electrophoresis. It is probable that the increased flexibility also serves to increase cyclization, as well as hybridization, of the inverted material with target DNA sequences.

Next, solutions of triethyleneglycol amidite **1** and levulinyl-protected triethyleneglycol phosphoramidite **2**, mixed in varying proportions (1:0.25, 1:1, 1:2, 1:4, and 1:6), were used for coupling to the support. This resulted in a variable proportion of sites on the support with levulinyl-protected hydroxyl groups. Remaining non-terminated sites were further reacted with reagent **3**, attaching a thymidine unit via a cleavable ester moiety. All non-standard phosphoramidites used in the present study were shown to couple with at least 98% efficiency. A mixed sequence oligonucleotide was synthesized on the above supports, detritylated, and then quantitatively phosphorylated in a 10 min reaction using *o*-chlorophenylphosphorobistriazolide (17). The phosphoromonotriazolide derivative was hydrolyzed to phosphodiester **4** (Fig. 2) using 0.3 M triethylammonium bicarbonate. While still on the machine, the support was treated with a buffered hydrazine solution to remove levulinyl ester, releasing the hydroxyl group (18).

The solid phase was removed from the cassette, suspended in pyridine, and treated for 120 min with the condensing agent MSNT (19). This results in coupling between 5′-phosphodiester functions and hydroxyl groups located on separate oligotriethyleneglycol chains, to form looped products **5**.

In order to monitor the inversion process, all components of the reaction mixture were released and combined for analysis. The supports were treated with 4-nitrophenylbenzaldoxime and tetramethylguanidine prior to aqueous ammonia treatment to reverse possible side-reactions caused by MSNT (19). The evaporated mixtures were treated with 80% aqueous acetic acid to release all remaining DNA fragments. All crude mixtures were desalted, dissolved in an equal volume of water, and analyzed by capillary electrophoresis. The electropherograms of the reaction products are presented in Figure 3.

All electropherograms show two major products. The peak appearing at 35.0 min represents non-inverted 5′-phosphorylated 19mers, as evidenced by co-migration with the authentic product (results not shown). The following peak at 36.2 min appears at the time expected for the inverted oligonucleotide **6**. This material is characterized by greater mass and/or charge, comparable to an oligonucleotide of 23–24 nt in length, which is the expected property of an inverted and cleaved oligonucleotide. An oligonucleotide of this size could not be formed as a result of any foreseeable side reaction; therefore, the existence of this material is taken to demonstrate inversion. The yield of inversion, defined as the fraction of inverted material **6** to the starting material **4**, reflects changes in the ratio between non-terminating **1** and terminating **2** amidites used in the early stage of synthesis. The increased inversion at higher ratios may be a consequence of the increased number of hydroxyls available for the phosphodiesters to react with, and of the lower density of oligonucleotides on the support, resulting in decreased steric hindrance of inversion. At the highest proportion of hydroxyls to phosphodiesters, inversion amounted to 72%. On the other hand, as is clear from comparison of the absolute capillary

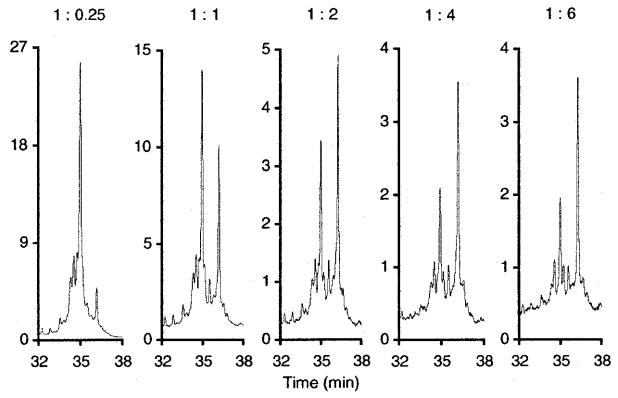

Figure 3. Effects on inversion efficiency by different proportions of protected sites on the supports during synthesis. Supports were treated with the indicated proportions of the non-terminating triethyleneglycol amidite and the terminating levulinyl-protected amidite, to yield variable numbers of protected sites before synthesis of the oligonucleotides. After *in situ* inversion, crude reaction products were removed from the support and analyzed by capillary electrophoresis.

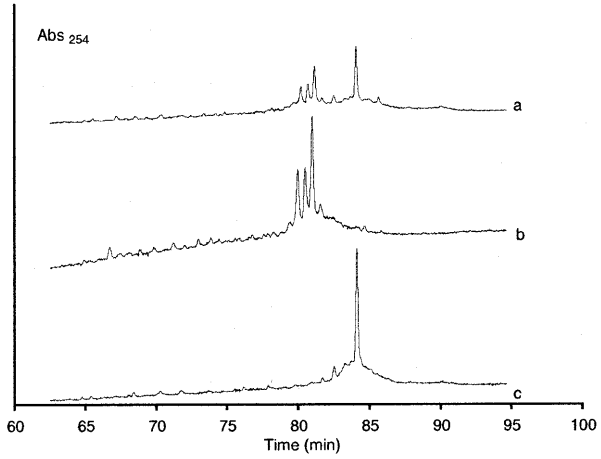

Figure 4. Products of the *in situ* inversion reaction, analyzed by capillary electrophoresis. (a) Total reaction products. (b) Material released from the support when the 3′-attachment was cleaved. (c) Molecules left on the support after the inversion and released for analysis.

electrophoresis peak sizes, the greatest total number of inverted oligonucleotides was formed at a 37.5% inversion yield on supports that had been treated with the terminating and non-terminating phosphoramidites at a 1:1 ratio. This amount of inverted oligonucleotide represents ~25% of the maximal amount of material that can be synthesized on this solid phase.

Quality of the inverted material

During solid phase oligonucleotide synthesis shorter oligonucleotide fragments are formed as a result of less than quantitative stepwise yields. The only commercially applied technology for *in situ* oligonucleotide synthesis results in formation of full-length material of on average only 5% purity for a typical 20mer, as calculated from the published stepwise coupling yields (20).

In our procedure only full-length oligonucleotides are functionalized for inversion, while truncated material is expected to be lost in the inversion since they fail to attach via the 5′-end. To investigate this, a mixed oligonucleotide sequence was synthesized and inverted *in situ*. After synthesis the support was divided into two aliquots. One was deprotected and oligonucleotides were cleaved from the support as described above to reveal all reaction products. The other aliquot was deprotected by treatment with ammonia, and all released products were collected and analyzed. These represent a mixture of molecules lacking one or more 5′-nucleotides, non-5′-phosphorylated oligonucleotides, and molecules of the correct composition that nonetheless failed to be inverted. The residual material was treated with aqueous acetic acid to cleave the phosphoramide bond, releasing inverted oligonucleotides. Results from capillary electrophoresis of these fractions are presented in Figure 4.

The total reaction mixture (Fig. 4, line a) reveals several peaks originating from the starting material and a single product corresponding to the inverted material. The ammoniacal fraction contains only truncated and other unreacted oligonucleotides (Fig. 4 line b). This step thus effectively removes all impurities from the final, solid phase-bound products. The remaining material is found in the fraction released under acidic condition (Fig. 4, line c), and represents essentially pure (>95%) intact inverted oligonucleotides. The traces of impurities present in this fraction most probably correspond to 3′-end fragments of depurinated material, inverted and then cleaved at the apurinic sites. However, any fragments of depurinated molecules that remain on the support would have 3′-phosphates, and therefore could not serve as primers in polymerase-dependent reactions. The inversion procedure presented herein would not be expected to remove any internally deleted oligonucleotides. However, the photolithographic method does not appear to result in an increased number of internally deleted fragments, since any molecules that fail to be deblocked to yield a 5′-hydroxyl are nonetheless rearranged to have permanently blocked 5′-ends, and thus cannot be extended in subsequent cycles (5). The *in situ* inversion process thus results in effective on-support oligonucleotide purification.

Primer extension from *in situ* inverted oligonucleotides

In situ inverted oligonucleotides present a free 3′-end that could be used to prime polymerase-dependent extension reactions. In a minisequencing reaction, the ability of DNA polymerases to selectively incorporate the complementary nucleotide triphosphate is used to discriminate between DNA sequence variants in a template.

A 19mer sequence was synthesized, inverted *in situ*, and left on the solid support to serve as a primer. Equal amounts of the support were used in four separate reactions with a synthetic oligonucleotide template hybridized to the inverted primer. *Taq* DNA polymerase and one of the four ^{3}H-labeled nucleotide triphosphates were added to each of the reactions. The template strand contained a cytosine residue opposite the position immediately downstream of the primer. As seen in Figure 5A, the inverted oligonucleotide served as a primer, and only the expected dGTP was incorporated. Applied in an array format with fluorescent dye-terminator nucleotides, inverted oligonucleotides could permit screening of large sets of DNA sequence variants.

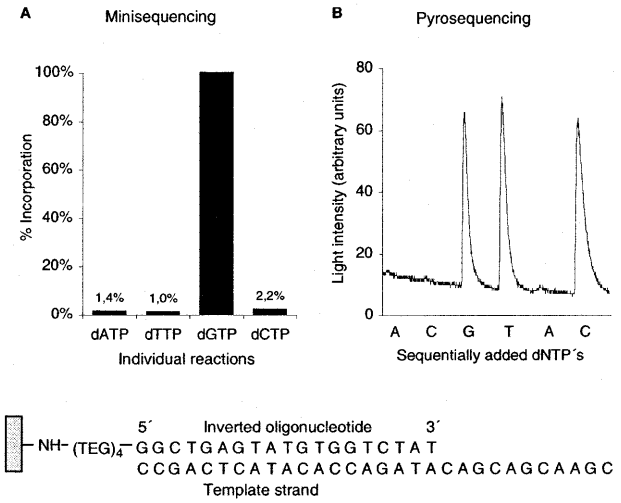

Figure 5. Sequence analysis by primer extension reactions from *in situ* inverted oligonucleotides. A template oligonucleotide was hybridized to the inverted oligonucleotide primer and extended by a DNA polymerase in the presence of the indicated nucleotide triphosphates. (**A**) Products of minisequencing reactions. (**B**) Results of pyrosequence analysis.

Oligonucleotides with free 3′-ends can also be used to monitor consecutive incorporation of nucleotides by a primer hybridized to a sequencing template in a process referred to as pyrosequencing (14). Here, individual nucleotide triphosphates are sequentially added and incorporated nucleotides are detected through the liberated pyrophosphate. Through an enzymatic reaction the pyrophosphate is converted into ATP and used as the energy source for the luciferase/luciferin system, generating a light signal. In this manner, sequences of a few tens of nucleotides can be determined without gel separation.

Figure 5B shows the result of a pyrosequencing reaction with the same inverted oligonucleotide primer and template as used in the minisequencing reactions. Addition of complementary nucleotides resulted in light signals that revealed the nucleotide sequence, while non-complementary nucleotides gave only a minimal increase over background.

SUMMARY AND CONCLUSIONS

The present *in situ* oligonucleotide inversion approach allows efficient solid phase oligonucleotide synthesis in the usual 3′→5′ direction, followed by conversion to material having a free 3′-end. Solid phase-bound oligonucleotides prepared according to this method can prime polymerase-mediated extension reactions, useful in a number of genetic tests. The method is suitable to produce high quality oligonucleotide arrays, and it is compatible with all existing methods for *in situ* oligonucleotide synthesis. The oligonucleotide inversion strategy requires some additional treatment of the support before and after oligonucleotide synthesis, but the oligonucleotide synthesis, whether by photolithography or physical masking, is unaltered. In contrast to other methods for *in situ* synthesis, failure sequences are removed in the present method. This should be helpful for all DNA microarray-based assays, whether based on oligonucleotide hybridization or primer extension by polymerases.

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