Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7510–7514, November 1985
Biochemistry

Modified oligonucleotides as alternatives to the synthesis of mixed probes for the screening of cDNA libraries

(triazolonucleosides/2-amino-2'-deoxyadenosine/hybridization probes/human antithrombin III)

TAM HUYNH-DINH*, NATHALIE DUCHANGE†, MARIO M. ZAKIN†, ANNE LEMARCHAND*, AND JEAN IGOLEN*

*Unité de Chimie Organique, Unité Associée n° 487, and †Unité de Biochimie Cellulaire, Unité Associée n° 517, Centre National de la Recherche Scientifique, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris, Cedex 15, France

Communicated by Andre Lwoff, July 15, 1985

ABSTRACT **Two simple alternatives to the synthesis of mixed oligodeoxyribonucleotide probes are described: mixed deblocking of triazolodeoxynucleosides for the A/G and C/T degeneracies or incorporation of 2-amino-2'-deoxyadenosines into a determined sequence for a higher stability of the hybridization duplexes. The synthetic oligodeoxyribonucleotides obtained were successfully tested in comparison with a classical mixed probe for their capacity to identify specific cDNA clones of human antithrombin III. The results are discussed with respect to the utilization of these synthetic oligonucleotides as hybridization probes for the isolation of cloned cDNA sequences.**

Mixed probes—i.e., mixtures of synthetic oligodeoxynucleotides representing all possible codon combinations for a short peptide sequence—have been developed for the screening of libraries of cloned cDNA (1). Although very complex mixtures of 384 (2) or 1024 (3) sequences were successfully applied to identifying cDNA clones or to site-directed mutagenesis experiments, problems of differential coupling rates during the chemical synthesis still occur: the equimolecular proportion of all the different oligonucleotides in the mixture cannot be guaranteed or even checked. Some approaches have been proposed to overcome these difficulties: 5' color-coded protecting groups (4, 5) and incorporation of "no-base" or phenyl analogues at points of redundancy (6).

Our approach to obtain simpler probes is based in the exploration of two methods; the first one consists in the incorporation of modified nucleosides in the probes, which after deblocking give a mixture of purines and pyrimidines at the sites of degeneracy. This method derives from previous work (7) in which we have shown that the 4-triazolothymidine (t^4T) or the 6-triazolo-2'-deoxyguanosine (t^6G) gave a mixture of thymidine and 5-methyl-2'-deoxycytidine (m^5C) or 2'-deoxyguanosine and 2-amino-2'-deoxyadenosine (n^2A), respectively, under definite conditions.‡ This procedure will allow a direct control of the coupling steps during the synthesis of one oligonucleotide that, after deblocking, will give a mixture of several sequences comparable to a mixed probe. The second method consists in the synthesis of a single sequence with a higher hybridization potential owing to the extra hydrogen bindings of the n^2A. By incorporating n^2A instead of A during the synthesis of a determined sequence, all of the A·T pairs (two hydrogen bondings) will be transformed into n^2A·T pairs (three hydrogen bondings), almost equivalent to a G·C base pairing.

In this paper we report the synthesis and the hybridization assays of modified oligonucleotides complementary to cloned antithrombin III (AT III) DNA sequences. Five probes were synthesized as indicated in Fig. 1, which shows the amino acid sequence of a segment of the AT III protein (11) and its corresponding mRNA sequence. Probe 1 is a classical mixed probe (1). Probe 2 results from a mixed deblocking (7), giving a mixture of thymidine and m^5C or 2'-deoxyguanosine and n^2A at the T/C or A/G sites, respectively. These sites correspond to positions 9, 12, and 15 in the original mRNA sequence. In probe 3, n^2A was incorporated instead of A at positions 2, 5, and 7 of the mRNA sequence. This probe presents a single mismatch with the original complementary sequence at position 12 (T instead of C). Probe 4 differs from probe 3 in that it contains A instead of n^2A at the corresponding positions. Probe 5 incorporates n^2A in the same positions as in probe 3 but includes three mismatches with the original sequence at positions 9, 12, and 15 (A instead of G, T instead of C, and C instead of T, respectively).

MATERIALS AND METHODS

Oligonucleotide Synthesis. The synthesis strategy and coupling yields are reported in Fig. 2. All oligonucleotides were manually synthesized by the solid-phase phosphotriester method (12) using triisopropylsulfonylnitrotriazole as coupling agent (13) on a 2-mmol scale. The modified nucleoside phosphotriesters t^4T, t^6G, and n^2A have been described (14–16).

Deblocking Steps. After completion of the synthesis, the resins carrying probes 1, 3, 4, and 5 were treated with (*i*) pyridine-2-aldoxime/tetramethylguanidine (1 M) in dioxane/water, 1:1 (vol/vol) for 15 hr at room temperature and (*ii*) concentrated ammonium hydroxide for 5 hr at 50°C. Supports 3 and 5 needed an additional deblocking step with ethylenediamine for 72 hr at room temperature (16). The resin containing probe 2 was treated with pyridine-2-aldoxime/tetramethylguanidine containing 0.3 M concentrated NH_4OH (1:10, vol/vol) for 15 hr at room temperature (7), with concentrated ammonium hydroxide for 5 hr at 50°C, and with ethylenediamine for 72 hr at room temperature.

Purification Step. The crude 5'-dimethoxytrityl-protected oligonucleotides were purified by preparative HPLC on a Zorbax ODS column (9.4-mm diameter) with a flow rate of 5.5 ml/min. Buffer A was 0.01 M triethylammonium acetate (pH 7.0); buffer B was buffer A containing acetonitrile (1:1, vol/vol). The linear gradient started at 10% buffer A and reached 50% buffer A/50% buffer B in 20 min.

After treatment with 80% acetic acid for 20 min at room temperature, the fully deprotected products were exchanged into the ammonium form on a Dowex NH_4^+ column.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AT III, antithrombin III; bp, base pair(s); t^4T, 4-triazolothymidine; t^6G, 6-triazolo-2'-deoxyguanosine; m^5C, 5-methyl-2'-deoxycytidine; n^2A, 2-amino-2'-deoxyadenosine.

‡Designations for the deoxyribonucleosides in the probe sequence omit the "d" for deoxy for simplicity.

Amino acid sequence	^{251} Met Met Tyr Gln Glu Gly^{256}
mRNA sequence	5′ AUG AUG UA$^{C}_{U}$ CA$^{A}_{G}$ GA$^{A}_{G}$ ^{16}G–3′
mRNA sequence found	5′ AUG AUG UAC CAG GAA G 3′
Probe 1 (mixed probe)	3′ TAC TAC AT$^{A}_{G}$ GT$^{C}_{T}$ CT$^{C}_{T}$ C 5′
Probe 2 (mixed deblocking)	3′ TAC TAC AT$^{*A}_{G}$ GT$^{*C}_{T}$ CT$^{*C}_{T}$ C 5′
Probe 3 (*A, one mismatch)	3′ TAC TAC ATG GTT CTT C 5′
Probe 4 (A, one mismatch)	3′ TAC TAC ATG GTT CTT C 5′
Probe 5 (*A, three mismatches)	3′ TAC TAC ATA GTT CTC C 5′

FIG. 1. Sequences of synthetic oligonucleotides complementary to a mRNA fragment of the human AT III as in this paper and in refs. 8–10. *A, n^2A; *C, m^5C; single underline, choice at the degeneracy site; double underline, mismatch.

5′ [^{32}P]Phosphorylation of Nucleotides. 5′ Phosphorylation with T4 polynucleotide kinase {100 pmol of oligonucleotide/50 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP/10 units of enzyme} and purification (20% polyacrylamide gel) were carried out as described (17). After electrophoresis, the labeled band was excised, crushed, and extracted overnight with water.

Source of DNA and Transformants. pATIII63, a recombinant plasmid of pAT153/PvuII/8 (18) containing the cDNA of human antithrombin III inserted in its *Pvu* II site, was isolated from a human liver cDNA library. The construction and the screening of this library have been described (19, 20).

Preparation and Hybridization of Colony Filters. Bacterial colonies were picked onto agar plates containing ampicillin at 50 μg/ml. Three pATIII63 clones and three Tf23 clones containing the cDNA of human transferrin (21) as a negative control were disposed on each plate. After overnight growth, colonies were transferred to Whatman 541 filters, amplified 20 hr on agar plates containing chloramphenicol at 250 μg/ml and prepared for hybridization as described by Gergen *et al.* (22).

Colony filters were prehybridized in 6× NET buffer (1× NET = 0.15 M NaCl/0.015 M Tris·HCl, pH 7.5/0.001 M EDTA) containing 0.5% Nonidet P-40 and 100 μg of both tRNA and sonicated salmon sperm DNA per ml for 2 hr at 42°C. Hybridization was performed in the same solution with 0.5×10^6 cpm/ml of 5′-[γ-^{32}P]ATP-phosphorylated oligonucleotide for 20 hr at 42°C. Filters were washed at room temperature for 1 hr with four changes of 6× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.15 M sodium citrate, pH 7.2) containing 0.1% $NaDodSO_4$ and were exposed to an x-ray film. Filters were then washed for 1 hr at 40°C with four changes of 6× NaCl/Cit containing 0.1% $NaDodSO_4$ and exposed a second time.

Thermal Denaturation. Spots containing 50 ng of pATIII63 DNA were immobilized on nitrocellulose filters as described (23). Hybridization was performed in 6× NaCl/Cit containing 5% dextran sulfate, 0.5×10^6 cpm of 5′-[γ-^{32}P]ATP-phosphorylated oligonucleotide (50 pmol) per ml, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin for 20 hr at room temperature. Filters were washed with several changes of 6× NaCl/Cit at 4°C until no radioactivity was eluted.

For thermal denaturation, 5 ml of 6× NaCl/Cit was placed

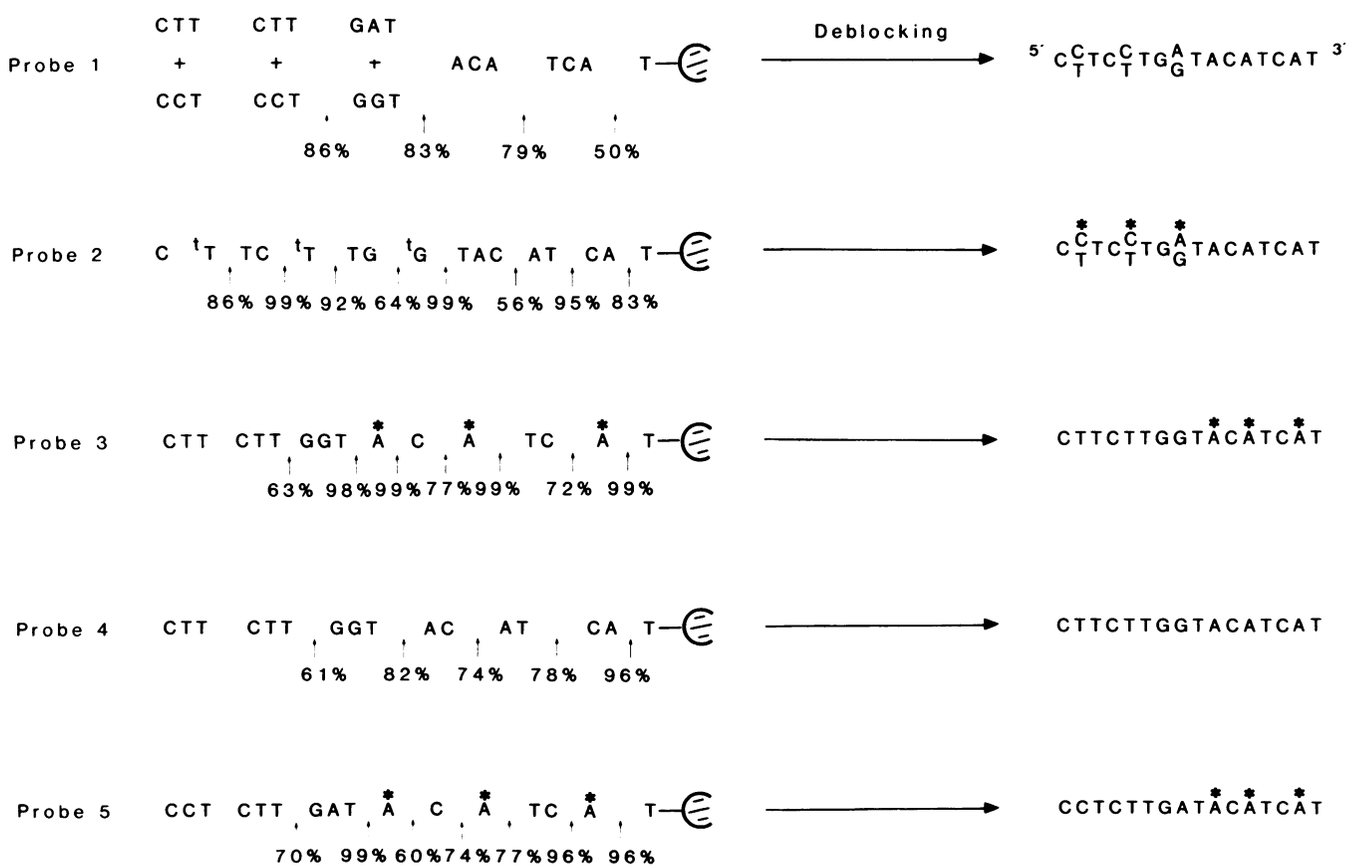

FIG. 2. Coupling yields of the synthesis of probes 1–5. *A, n^2A; *C, m^5C; ^{t}T, t^4T; ^{t}G, t^6G.

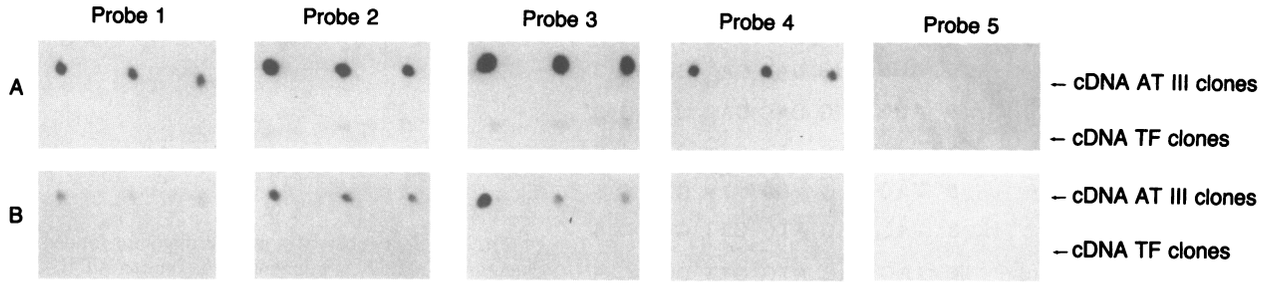

FIG. 3. Colony hybridization. Each of the five 5′ γ-^{32}P-labeled oligonucleotides (probes 1–5 of Fig. 1) was hybridized to three clones containing a cDNA of human AT III and to three clones containing a cDNA of human transferrin (TF; negative control) transferred to Whatman 541 filters. Filters were hybridized 20 hr at 42°C and washed 1 hr in 6× NaCl/Cit containing 0.1% $NaDodSO_4$ at room temperature (*A*) and at 40°C (*B*).

on each filter, and the temperature was raised. After 1 min at a defined temperature, liquid was removed, and its radioactivity was measured.

RESULTS

Isolation of the cDNA of Human AT III. A mixed synthetic oligonucleotide probe (probe I of Fig. 1) corresponding to the amino acids 251 to 256 of human AT III (11) was used to screen a human liver cDNA library (see *Materials and Methods*). Sixteen recombinant clones out of 170,000 tested gave a positive signal of hybridization. Their cDNA content was characterized by restriction endonuclease mapping and DNA sequencing. The recombinant pATIII63 was selected for further studies. Sequence experiments showed that its insert was 1,600 base pairs (bp) encoding the total AT III protein sequence and the 5′ and 3′ flanking regions. The complete nucleotide sequence of this cDNA is essentially identical to the sequences previously reported (8–10).

Comparison of the Hybridization Pattern of the AT III cDNA with the Different Probes. Probes 2–5 were tested for their capacity to identify cDNA clones of AT III transferred to Whatman filters. In these experiments, probe 1 was used as a positive control. As seen in Fig. 3*A*, probes 1–4 gave a positive signal with AT III cDNA clones when filters were washed after hybridization under nonstringent temperature conditions. Probe 5 did not exhibit any significant signal. Three unrelated clones containing the cDNA of human transferrin (21) were included as negative controls. Washing under more stringent conditions indicated that only probe 4 was removed from the filters (Fig. 3*B*).

Thermal Denaturation. In order to study the thermal stability of the different oligonucleotide·cDNA AT III duplexes, probes 1, 2, 3, and 4 were hybridized to a fixed quantity of pATIII63 DNA immobilized on nitrocellulose filters, and the radioactivity that was eluted at different washing temperatures was measured (Fig. 4). Probes 1, 2, and 3 exhibited similar patterns of thermal denaturation [melting temperature (t_m) = 43°C]. The increased hybridization stability of probe 3 compared to probe 4 (t_m = 37°C) shows the effect of the n^2A_d residues. This result agrees with the fact that only probe 4 was eliminated under stringent washing conditions in the Whatman filter experiments (see above and Fig. 3*B*).

Screening of a Human Liver cDNA Library. On the basis of the results obtained above, we decided to test the ability of probes 1, 2, and 3 to recognize specific cDNA clones of AT III in a human liver cDNA library. An aliquot of the human liver cDNA library previously screened with probe 1 was plated, and 1000 clones, containing a positive clone of AT III cDNA, were screened with probes 1, 2, and 3. Only the identified AT III cDNA clone gave a strong positive hybridization signal (see Fig. 5). This indicates that no positive clone is present in the other 999 colonies tested. This negative result is not surprising because the frequency of the AT III cDNA recombinant clones in the library was 1 in 10,000 (see above). This result confirms the specificity of the hybridization signal obtained with the three synthetic probes.

DISCUSSION

Mixed oligonucleotide probes have been used for the screening of libraries of cloned cDNA (1). In order to overcome the difficulties encountered in the synthesis of these probes, a more simple approach is presented in this paper. Four different oligonucleotides (probes 2, 3, 4, and 5; Fig. 1) were synthesized in addition to a classical mixed probe (number 1, Fig. 1).

The design of probe 2 is directly derived from previous work (7) in which we have shown that, under very definite conditions, the t^4T or t^6G gave a mixture of thymidine and m^5C or 2′-deoxyguanosine and n^2G, respectively, in approx-

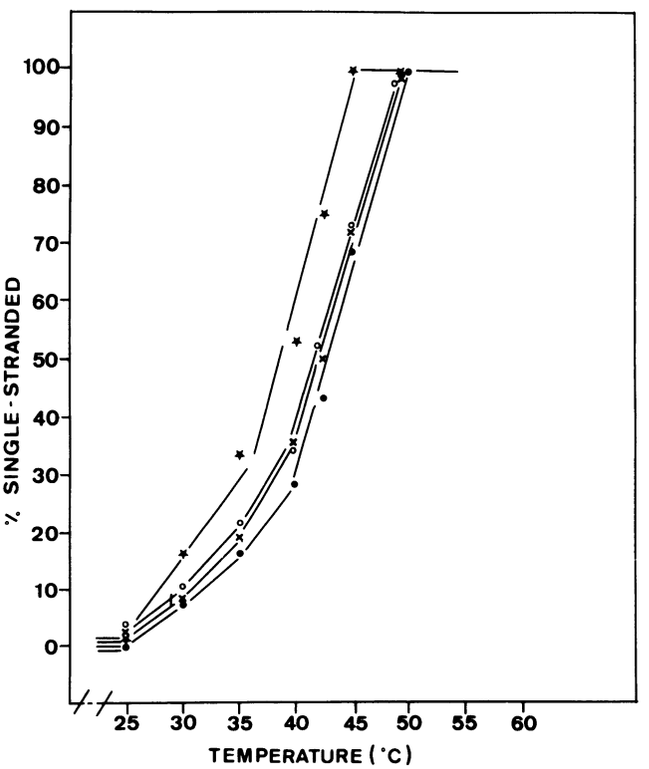

FIG. 4. Thermal denaturation. Probes 1 (×), 2 (●), 3 (○), and 4 (★) were hybridized to pATIII63 cDNA immobilized on nitrocellulose filters. After extensive washings at 4°C, filters were subjected to various washes at defined temperatures. The radioactivity eluted at each temperature is plotted as the fraction of the total probe becoming single-stranded.

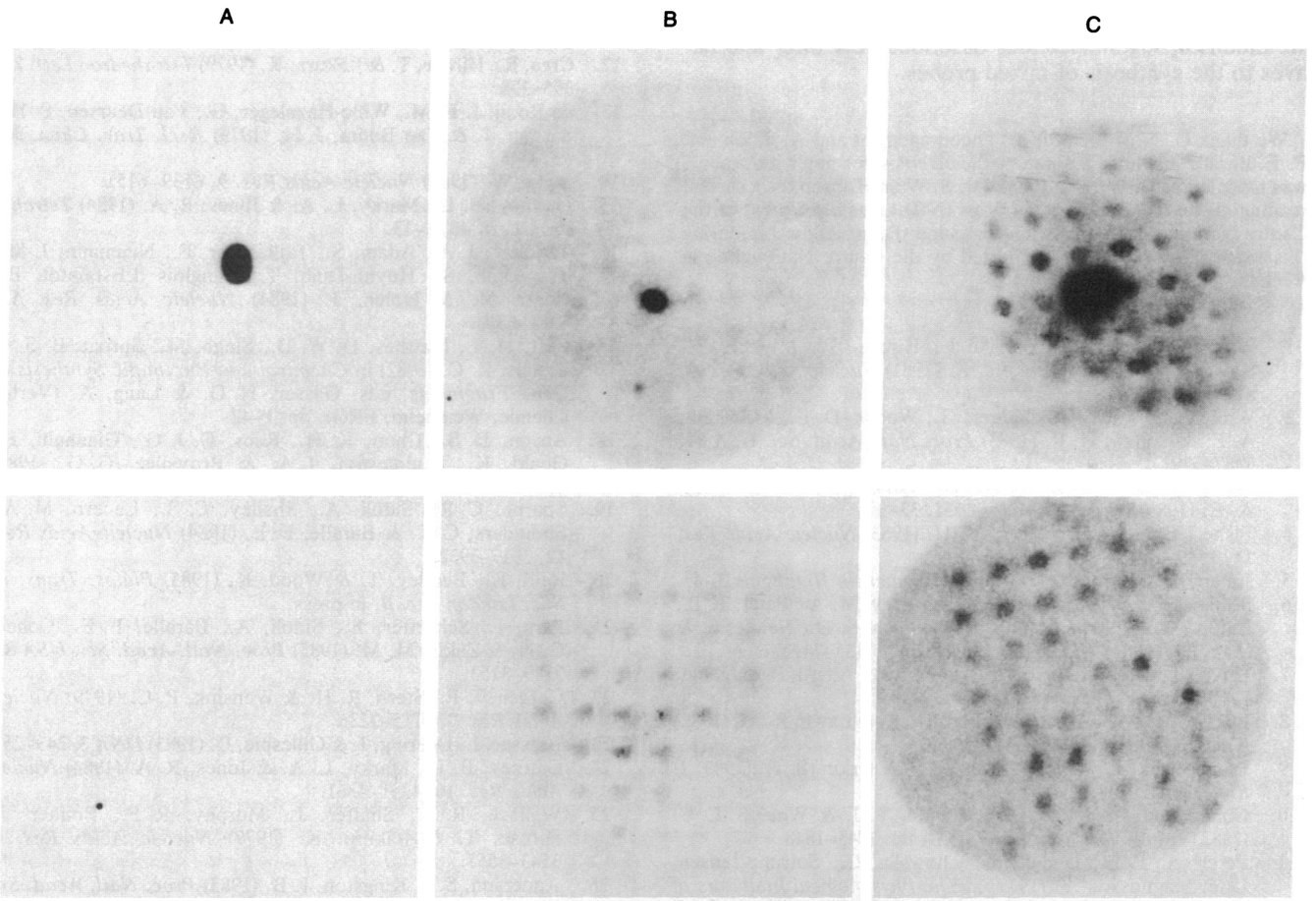

FIG. 5. Hybridization of the oligonucleotide probes to AT III cDNA- and non-AT-III-cDNA-containing colonies from a human liver cDNA library. Similar filters were screened with probe 1 (*A*), probe 2 (*B*), and probe 3 (*C*). (*Upper*) Filters with a positive clone. (*Lower*) Filters with only negative clones.

imately equal proportions. The fact that probes 1 and 2 present the same thermal denaturation curve (Fig. 4) was expected because, in these two probes, which differ only by their ways of synthesis, only the perfect complementary sequence (one out of eight) can give the duplex under stringent washing conditions. The similar hybridization properties of probe 2 compared to the mixed probe 1 show that the two triazolonucleosides can replace the use of mixtures of monomers (in manual or automatic synthesis) or trimers (1) (in the phosphotriester method) for the synthesis of a hybridization probe. Under these conditions, efficiency of the couplings can be followed until the end of the synthesis of the probe.

As demonstrated by several spectroscopic studies (15, 24) the n^2A·T pairs seem more stable than A·T pairs but less stable than C·G pairs (15). Therefore, probe 3 was synthesized with n^2A instead of A (positions 2, 5, and 7 of the original mRNA sequence) in addition to the choice of a G at the G/A site (position 9) and T at the T/C site (position 15). The final structure of this oligonucleotide presents one mismatch (T instead of C at position 12) with the mRNA sequence. Probe 4 has the same sequence as probe 3, but was synthesized with A instead of n^2A. This was done in order to check the usefulness of n^2A in the hybridization experiments. Probe 4 does not give a stable duplex (25) with the cDNA for AT III, while the same sequence with n^2A (probe 3) shows a stable positive response (Figs. 2, 3, and 4). Analysis of Fig. 5 shows that, under the same washing conditions, probe 3 presented a higher background than did probes 1 and 2 when a human cDNA library was screened. More stringent washing procedures can attenuate this background, but the strong positive signals obtained over the background made it unnecessary. The synthetic oligonucleotide numbered 5 (Fig. 1) contains three n^2A residues (as probe 3) and presents three mismatches with the original mRNA sequence (positions 9, 12, and 15; Fig. 1). The comparison of its hybridization properties with that of probe 3 indicates that the extra stability of three n^2A·T pairs cannot overcome the three mismatches in the same sequence (Fig. 3*A*). This also indicates that insertion of n^2A into a sequence should not give a false positive response. Therefore, the specificity of probe 3 is proved, and the n^2A appears as a very potent candidate for another type of hybridization probe. In combination with a simple choice of nucleosides at the degeneracy sites, it will be possible to synthesize and purify, a single sequence that possesses the hybridization potential of a mixture of oligonucleotides. More experiments are needed to assess the usefulness of the n^2A alternative as compared to the present tendency to use very long (>40 bp) synthetic DNA probes (26) for screening genomic libraries. Very long DNA probes need an enzymatic ligation or the costly access to an automatic DNA synthesizer, whereas short (15–20 bp) oligonucleotides with n^2A could be more easily and cheaply prepared, even by nonspecialists. The main advantage of the n^2A alternative lies in the difficult cases where only short fragments of the protein have been sequenced. Another interest in the replacement of an A by n^2A in synthetic oligonucleotides will be in directed mutagenesis experiments, by allowing the formation of a stable duplex with primer-containing mismatches.

In conclusion, we have demonstrated that probes 2 and 3 are valid options to the classical mixed probes in the

identification of cDNA clones of human AT III. Therefore, the modified oligonucleotides described here offer alternatives to the synthesis of mixed probes.

We thank G. N. Cohen for his encouragement and A. Sidoli and F. E. Baralle in whose laboratory the cDNA screening with probe 1 was made by N.D. We also thank Dr. S. Wain-Hobson for a careful reading of the manuscript. One of us (N.D.) was a recipient of the Centre National de Transfusion Sanguine (Paris) fellowship during this work. This work was supported by the Centre National de la Recherche Scientifique.

1. Wallace, R. B., Johnson, M. J., Hirose, T., Miyake, T., Kawashima, E. H. & Itakura, K. (1981) *Nucleic Acids Res.* **9,** 879–894.
2. Whitehead, A. S., Goldberger, G., Woods, D. E., Markham, A. F. & Colten, H. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5387–5391.
3. Kalderon, D., Richardson, W. D., Markham, A. F. & Smith, A. E. (1984) *Nature (London)* **331,** 33–38.
4. Fisher, E. H. & Caruthers, M. H. (1983) *Nucleic Acids Res.* **11,** 1589–1599.
5. Jinicny, J. & Jones, M. B. (1984) *Cruachem Highlights* 3–4.
6. Millican, T. A., Mock, G. A., Chauncey, M. A., Patel, T. P., Eaton, M. A. W., Gunning, J., Cutbush, S. D., Neidle, S. & Mann, J. (1984) *Nucleic Acids Res.* **12,** 7435–7453.
7. Huynh-Dinh, T., Langlois d'Estaintot, B., Allard, P. & Igolen, J. (1985) *Tetrahedron Lett.* **26,** 431–435.
8. Bock, S. C., Wion, K. L., Vehar, G. A. & Lawn, R. M. (1982) *Nucleic Acids Res.* **10,** 8113–8125.
9. Prochownik, E. V., Markham, A. F. & Orkin, S. H. (1983) *J. Biol. Chem.* **258,** 8389–8394.
10. Chandra, T., Stackhouse, R., Kidd, V. J. & Woo, S. L. C. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1845–1848.
11. Petersen, T. E., Dudekwojciechowska, G., Sotrupp-Jensen, L. & Magnusson, S. (1979) in *The Physiological Inhibitors of Coagulation and Fibrinolysis*, eds. Collen, D., Wiman, B. & Verstraete, M. (Elsevier/North-Holland Biomedical, Amsterdam), pp. 43–54.
12. Crea, R., Hirose, T. & Itakura, K. (1979) *Tetrahedron Lett.* **20,** 395–398.
13. de Rooij, J. F. M., Wille-Hazeleger, G., Van Deursen, P. H., Serdijn, J. & Van Boom, J. H. (1979) *Recl. Trav. Chim.* **98,** 537–548.
14. Sung, W. (1981) *Nucleic Acids Res.* **9,** 6139–6151.
15. Gaffney, B. L., Marky, L. A. & Jones, R. A. (1984) *Tetrahedron Lett.* **40,** 3–13.
16. Taboury, J. A., Adam, S., Taillandier, E., Neumann, J.-M., Tran-Dinh, S., Huynh-Dinh, T., Langlois d'Estaintot, B., Conti, M. & Igolen, J. (1984) *Nucleic Acids Res.* **12,** 6281–6305.
17. Gait, M. J., Matthes, H. W. D., Singh, M., Sproat, B. S. & Titmas, R. C. (1982) in *Chemical and Enzymatic Synthesis of Gene Fragments*, eds. Gasser, H. G. & Lang, A. (Verlag Chemie, Weinheim, FRG), pp. 1–42.
18. Anson, D. S., Choo, K. H., Rees, D. J. G., Giannalli, F., Gould, K., Huddleston, J. A. & Brownlee, G. G. (1984) *EMBO J.* **3,** 1053–1060.
19. Sharpe, C. R., Sidoli, A., Shelley, C. S., Lucero, M. A., Shoulders, C. C. & Baralle, F. E. (1984) *Nucleic Acids Res.* **12,** 3917–3932.
20. Reid, K., Bentley, T. & Wood, K. (1985) *Philos. Trans. R. Soc. London Ser. B*, in press.
21. Park, I., Schaeffer, E., Sidoli, A., Baralle, F. E., Cohen, G. N. & Zakin, M. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3149–3153.
22. Gergen, J. P., Stern, R. H. & Wensink, P. C. (1979) *Nucleic Acids Res.* **7,** 2115–2136.
23. Bresser, J., Doering, J. & Gillespie, D. (1983) *DNA* **3,** 243–253.
24. Gaffney, B. L., Marky, L. A. & Jones, R. A. (1984) *Nucleic Acids Res.* **10,** 4351–4361.
25. Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T. & Itakura, K. (1979) *Nucleic Acids Res.* **6,** 3543–3557.
26. Anderson, S. & Kingston, I. B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6838–6842.