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(54) Title: CHIMAERIC OLIGONUCLEOTIDES AND USES THEREOF IN THE IDENTIFICATION OF ANTISENSE BINDING SITES**(57) Abstract**

A chimaeric oligonucleotide library for use in identifying an antisense binding site in a target mRNA, comprising a plurality of distinct chimaeric oligonucleotides capable of hybridizing to mRNA to form a duplex, the nucleotide sequences of which each have a common length of 7 to 20 bases and are generated randomly or generated from information characterising the sequence of the target mRNA, wherein substantially all the nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library, and wherein each nucleotide sequence comprises: a) a recognition region comprising a sequence of nucleotides which is recognisable by a duplex-cutting RNAase when hybridized to the mRNA, and b) a flanking region comprising a sequence of chemically-modified nucleotides which binds to the mRNA sufficiently tightly to stabilise the duplex for cutting of the mRNA in the duplex by the duplex-cutting RNAase, wherein the nucleotides constituting the flanking region are different from those constituting the recognition region, and wherein each oligonucleotide is protected against exonuclease attack.

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CHIMAERIC OLIGONUCLEOTIDES AND USES THEREOF IN THE IDENTIFICATION OF ANTI-SENSE BINDING SITES**Field of the Invention**

The present invention relates to an oligonucleotide library, more particularly a chimaeric oligonucleotide library, and uses thereof in the identification of antisense binding sites in target mRNA and in providing potential therapeutic agents.

Background to the Invention

Antisense oligonucleotides are single-stranded nucleic acids which are complementary to the coding or "sense" strand of genetic material. An antisense oligonucleotide is therefore also complementary to the mRNA produced from the genetic material. If antisense DNA or RNA is present in a cell with the mRNA, hybridisation takes place to form a duplex thereby preventing translation of the mRNA by ribosomes to make a protein. Thus, antisense RNA can be used to block the expression of genes that make proteins.

The antisense approach to the inhibition of gene expression, though conceptually straightforward, presents technologically demanding challenges. A variety of approaches have been taken by various academic groups and biotechnology companies. Oligonucleotides have been made with sugar modifications, such as 2'-O allyl ribonucleotides, and with backbone modifications in the phosphate group, such as phosphorothioate deoxyribonucleotides. However, production of these individual oligonucleotides for application as antisense therapeutics, reagents or tools for drug target validation has been hampered because methods of identifying potentially efficacious antisense compounds against a given target mRNA are extremely difficult. Even with an mRNA of known sequence, it is often impossible to predict what sub-sequences in the target mRNA might be available for antisense binding because of the three-dimensional structure of the mRNA and the association of RNA with proteins.

In an alternative approach to the use of chemically modified oligonucleotides, Lieber and Strauss (ref 22) report the use of

a ribozyme expression library for the purpose of selecting cleavage sites in target RNAs. The ribozyme approach suffers from the disadvantage that it requires cleavage sites containing GUC or CUC and thus is not generally applicable to all possible cleavage sites. In addition, cleavage efficiency is relatively low, and chemical synthesis of ribozyme libraries is difficult.

Summary of the Invention

According to one aspect, the present invention provides a chimaeric oligonucleotide library for use in identifying an antisense binding site in a target mRNA, which comprises a plurality of distinct chimaeric oligonucleotides capable of hybridizing to mRNA to form a duplex, the nucleotide sequences of which each have a common length of 7 to 20 bases and are generated randomly or generated from information characterising the sequence of the target mRNA, wherein substantially all the nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library, and wherein each nucleotide sequence comprises:

a) a recognition region comprising a sequence of nucleotides which is recognisable by a duplex-cutting RNAase when hybridized to the mRNA, and

b) a flanking region comprising a sequence of chemically-modified nucleotides which binds to the mRNA sufficiently tightly to stabilise the duplex for cutting of the mRNA in the duplex by the duplex-cutting RNAase, wherein the nucleotides constituting the flanking region are different from those constituting the recognition region; and wherein each oligonucleotide is protected against exonuclease attack.

The mRNA may be from human or other mammalian origin or from invertebrates. The term mRNA as used herein also encompasses the corresponding RNA from plants, viruses and bacteria.

Chimaeric Oligonucleotide

Each chimaeric oligonucleotide forming the library may be made synthetically using any commonly-available oligonucleotide

sequence synthesizer. The exact length of the nucleotide sequence will reflect a balance between achieving the necessary specificity and keeping the length to a minimum to minimise cost. Preferably, the nucleotide sequence has a length in the range 10 to 20, more preferably 14 to 17 bases, yet more preferably around 15 bases.

The oligonucleotide is preferably protected against nuclease attack so as to minimise degradation in the cell and increase its stability. This is particularly important in the design of an antisense compound for therapeutic use. Protection against exonuclease attack may be achieved by protecting one or preferably both ends of the oligonucleotide, for example by reverse T or any other well-known method. Selection of the nucleotides constituting the recognition and flanking regions may also contribute to stability against nuclease because some nucleotides are more nuclease-resistant than others.

Preferably, each chimaeric oligonucleotide comprises two flanking regions, one on either side of the recognition region. In this way, the recognition region may be thought of as a "window" flanked by the two flanking regions so as to form with the mRNA a substrate for the duplex-cutting RNAase. In a preferred embodiment, each of the flanking regions is protected against exonuclease attack, preferably by reverse T. A preferred duplex-cutting RNAase is RNAase H, advantageously endogenous RNAase H (Ref 23).

The nucleotides constituting the recognition region are either modified or unmodified nucleotides and are preferably deoxyribonucleotides or phosphorothioate deoxyribonucleotides (see Figure 4c). These nucleotides are recognisable by RNAase H when hybridized to mRNA. Typically, the recognition region comprises at least four nucleotides, preferably 5 to 10 nucleotides. In a particularly preferred embodiment, the recognition region comprises five nucleotides.

The nucleotides constituting the flanking region are chemically modified so as to increase the binding constant of the oligonucleotide for hybridization to the target mRNA and preferably to increase stability of the oligonucleotide *in vivo*. For a particular antisense oligonucleotide, the efficiency of hybridization to mRNA is a function of concentration. Thus, to improve hybridization at a given concentration, the stability of the hybrid duplex must be increased. A number of chemical modifications can be introduced into the oligonucleotide for this purpose and these fall into three broad classes (see also Figure 1, regions 1, 2 and 3):

Sugar Modifications

Various modifications to the 2' position in the sugar moiety may be made. For example, both 2'-O methyl oligoribonucleotides and 2'-O allyl oligoribonucleotides may be useful (see references 1 and 2 and see also Figure 2a and b). These analogues do not form hybrid duplexes with RNA which are substrates for RNAase H. In a particularly preferred embodiment of the present invention, two flanking regions, each having four or five of one of the modified sugar-containing oligoribonucleotides, flank a window region of four or five normal deoxyribonucleotides. The window region will thereby allow cleavage of the mRNA and the sugar-modified flanking regions increase the binding of the chimaeric oligonucleotide to the mRNA. Other 2' sugar modifications which may be used include F-substituted and NH₂-substituted oligoribonucleotides (see Figure 2c and 2d and references 3 and 4).

Base Modifications

The chemically-modified nucleotides constituting the flanking region may be modified in the base moiety. The propyne analogues of dT and dC, 5-propynyl deoxyuridine (see Figure 3a) and 5-propynyl deoxycytidine (see Figure 3b), both increase the duplex hybridization temperature and stabilize the duplex. This stabilization may be due to increased strength of hydrogen bonding to each Watson-Crick partner or increased base stacking

(or both). 2-amino adenine is an analogue of dA (see Figure 3c) and also increases the stability of the duplex. This may be due to the formation of a third hydrogen bond with thymine. The 2-amino adenine-thymine base pair is intermediate in stability between a G.C and a A.T base pair.

Phosphate Modifications

The chemically-modified nucleotides constituting the flanking region may be modified in the phosphate moiety. Under certain conditions such as low salt concentration, analogues such as methylphosphonates (Figure 4a), triesters (Figure 4b) and phosphoramidates (Figure 4e) have been shown to increase duplex stability. The hybrid duplexes are not substrates for RNAase H. Further phosphate modifications include phosphorodithirates (Figure 4d) and boranophosphates (Figure 4f), each of which increase the stability of oligonucleotides.

Isosteric replacement of phosphorus by sulphur gives nuclease resistant oligonucleotides (see reference 14). Replacement by carbon at either phosphorus or linking oxygen is also a further possibility (see Figure 5).

In use, the chimaeric oligonucleotide of the invention acts as an antisense compound by specifically binding to target mRNA at an antisense binding site so that cleavage or cutting of the mRNA by a duplex-cutting RNAase takes place there. The chimaeric oligonucleotide will bind to the target mRNA to form a duplex. The recognition region is recognised by a duplex-cutting RNAase. The flanking region renders the duplex sufficiently stable to enable the RNAase to cut the mRNA in the duplex efficiently. Once cut, the mRNA and the oligonucleotide detach thereby leaving the oligonucleotide to bind a further mRNA. In this way, the chimaeric oligonucleotide acts catalytically.

Oligonucleotide Libraries

In a further aspect, the present invention provides use of an oligonucleotide library in a method of identifying an antisense

binding site in a target mRNA, wherein the oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases, preferably 10 to 20 bases, and each of which comprises a substrate for a duplex-cutting RNAase if hybridised to the mRNA, which library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library. The nucleotide sequences may comprise modified nucleotides, such as phosphorothioates, as described herein. The nucleotide sequences may be chimaeric or non-chimaeric.

In one embodiment, the library is generated randomly by means of an oligonucleotide sequence synthesizer. An aim of generating the sequences randomly is that substantially all possible nucleotide sequences of the specified length are generated. For a sequence of 10 bases in length (a 10-mer), 4^{10} distinct nucleotide sequences would need to be generated to cover all possibilities. This works out as approximately 10^6 distinct nucleotide sequences. For a 15-mer, the library would need approximately 10^9 to 10^{10} nucleotide sequences. Each nucleotide sequence will have a common length (*i.e.* they will all be 10-mers or will all be 11-mers, etc.). Any commonly-available oligonucleotide sequence synthesizer may be used for this purpose such as supplied by Applied Biosystems. All four possible bases are fed into the machine with an appropriate program using suitable nucleotides or modified nucleotides.

In an alternative embodiment, instead of generating the nucleotide sequences randomly they are generated from information characterising the sequence of the target mRNA. The sequence of the target mRNA needs to be known and can then be programmed into the oligonucleotide sequence synthesizer. For example, in the case of a gene which produces an mRNA of 450 nucleotides, a library of 15-mers would be produced with a total of 436 distinct nucleotide sequences (*i.e.* length of mRNA minus length of

nucleotide sequence plus 1). In this way, all potential sub-sequences of the mRNA would be represented in the library. This is advantageous over the random generation of the library because there is no dilution of potentially useful nucleotide sequences by randomly generated sequences not present in the target mRNA. A further way of ensuring that all sub-sequences of the mRNA are present in the library is to produce, in the case of an mRNA of 450 nucleotides, a library of 30 15-mers (i.e. length of mRNA divided by the length of the nucleotide sequence).

In a further aspect, the present invention provides a method of identifying an antisense binding site in a target mRNA, which comprises:

- 1) incubating with the target mRNA an oligonucleotide library and a duplex-cutting RNAase under conditions to produce target mRNA cut at the antisense binding site; and

- 2) identifying the antisense binding site from the position of the cut in the mRNA; wherein the oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases, preferably 10 to 20 bases, and each of which comprises a substrate for the duplex cutting RNAase if hybridized to the mRNA; and wherein the oligonucleotide library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of such common length which are present as sub-sequences in the target mRNA are present in the library.

Use of an oligonucleotide library in this manner enables identification of one or more antisense binding sites in a target mRNA and such identification can be achieved very rapidly in comparison with known methods. No information about the three-dimension structure of the mRNA is required because the identification of the antisense binding sites is empirical. Incubation of the target mRNA with the oligonucleotide library and the duplex-cutting RNAase can, by suitable variation of the reaction conditions, produce target mRNA cut at one or more

antisense binding sites. This is because the library will contain one or more oligonucleotides which are complementary to such binding sites and will bind thereto under appropriate conditions to form a duplex. The duplex acts as a substrate for the duplex cutting RNAase. When the mRNA is cut at the binding site the oligonucleotide is released and is thereby made available for further binding. The oligonucleotide therefore acts catalytically and the duplex-cutting RNAase acts enzymatically. The duplex-cutting RNAase is separate from the oligonucleotide library and is preferably from a cell extract. Preferably, the duplex-cutting RNAase is RNAase H. The target mRNA is also preferably from a cell extract. Advantageously, therefore, both RNAase H and mRNA are present in the same cell extract with which the oligonucleotide library is incubated.

The position of the cut in the mRNA may be determined by sequencing isolated cut target mRNA. Preferably, the cut target mRNA is amplified prior to isolation, for example by reverse transcription and polymerase chain reaction.

Once the antisense binding site is identified from the position of the cut in the mRNA and sequenced, an antisense oligonucleotide may be synthesized which is capable of binding to the site. In this way, a method is provided for the production of an antisense oligonucleotide. Thus, a chimaeric oligonucleotide of the type discussed above can be obtained. An important use of such a chimaeric oligonucleotide is as a therapeutic agent capable of hybridising to a specific antisense binding site in a target mRNA. The nucleotide sequence of the chimaeric oligonucleotide needs to be specific to the antisense binding site for this purpose.

In summary, using the library approach described herein, optimal sequences of effective antisense compounds can be identified against specific mRNA targets. The antisense compounds are useful as potential therapeutics, as tools for drug target validation, in diagnostics and as a research reagent.

The present invention will now be described in further detail, by way of example only, with reference to the accompanying drawings in which:

Figure 1 shows a generalised structure for nucleotides;
Figure 2 shows a generalised structure for sugar moieties present in nucleotides;
Figure 3 shows generalised structures of modified base moieties in nucleotides;
Figure 4 shows generalised structures of modified phosphate moieties in oligonucleotides;
Figure 5 shows isosteric modifications of the phosphate moieties in oligonucleotides;
Figure 6 shows a schematic representation of a method for identifying antisense binding sites from cut mRNA,
Figures 7a and 7b show calibration graphs of fluorescence against amounts of standard and sample oligonucleotides; and
Figures 8 to 12 show computer predictions of the secondary structure of TNF- α mRNA and their cut sites.

Nucleotide modifications

Figure 1 shows a generalised structure for nucleotides in which base 2 is connected to sugar 1 and phosphate 3 links the sugar to the next sugar in the sugar phosphate backbone. According to Figure 2, substituent group A may be O-alkyl, aryl or alkaryl (in particular O-Me) (see Ref 1). Alternatively, substituent group A may be O-allyl (Ref 2), F (Ref 3), or NH₂ (Ref 4).

The bases shown in Figures 3a, 3b and 3c are discussed respectively in References 5, 6 and 7. Referring to Figure 4, various phosphate modifications may be made, as summarised in the Table I below.

x	y	Ref
CH ₃	O	8
OR	O	9
S	O	10
S	S	11
NHR	O	12
BH ₃	O	13

R = any alkyl

Referring to Figure 5, the phosphate moiety may be replaced in accordance with the following Table II

X	Y	Z	Ref
O	CH ₂	O	15
O	CH ₂	S	16
S	CH ₂	O	17
O	C=O	O	18
O	C=O	NH	19
CH ₂	NH	O	20
CH ₂	NCH ₃	O }	21
CH ₂	O	NCH ₃ }	

Example 1

Referring to Figure 6, the following illustrates a preferred method for identifying an antisense binding site in a target mRNA and for producing an antisense chimaeric oligonucleotide directed to that binding site (see also Ref. 22).

A chimaeric oligonucleotide library of the type described herein is incubated with an mRNA substrate. In this example, the target mRNA is TNF- α mRNA in a cellular extract. Incubation takes between one and three hours. As shown in step 1 of Figure 6, two cut sites are produced at yields of 0.15% and 0.05% respectively. Most (99.8%) of the target mRNA is uncut. In this step, N and n each represent different nucleotides, as discussed above in the section on nucleotide chemistry. The library is either

completely random (*i.e.* of the order of 10^9 species) or specific to the target TNF- α (*i.e.* of the order of 1600 species).

According to step 2, a first cDNA strand is synthesized by using reverse transcriptase (RT). Oligo dT is used as a primer because the mRNA fragment has a poly A tail. Thermal denaturation under alkaline conditions yields a single strand of the first cDNA. According to step 3, the first strand cDNA is tailed with dC or dG to yield at least 10, usually 10 to 100 bases. All products (including both target mRNA and non-target mRNAs) now have a dT and a dC or dG tail.

In step 4, primers specific to the gene in question are designed to a region 5' of the poly A site. Using primers complementary to the gene and the dG (dC) tails, PCR is carried out to amplify full length target mRNA and cut target mRNA products.

In step 5, the PCR products are analysed by separation or polyacrylamide gel electrophoresis. In this way, cut products are separated from full length target mRNA by size. Cut products are identified as small bands and can be purified by gel purification. At this stage, cutting efficiency of the RNAase H can be measured by observing the intensity of the staining (*e.g.* on ethidium bromide stained agarose gels). Efficacy of the potential antisense drug depends upon cutting efficiency. Thus, at this stage, the dominant bands will identify products which have been cut most effectively by RNAase H and which may be efficacious. It is these bands which are selected for cloning in a bacterial vector such as Bluescript.

According to step 6 of Figure 6, TA cloning of cut fragments is effected using a T vector having a 3' terminal thimidine. Cut fragments are amplified by PCR and a single deoxyadenosine (dA) is added during the PCR reaction. In order to identify the specific recognition sequences of the effective compounds, the cloned cut RT PCR products are sequenced. The ends of the products are sequenced by dideoxysequencing and the specific

sites should follow the poly G or poly C series produced in step 3. The partial sequence of each antisense binding site is thus identified in this manner and the complete sequence of the antisense binding site is deduced from a sequence data bank. The complete sequence information is used to synthesize a suitable oligonucleotide (for instance a further chimaeric oligonucleotide) which can then be tested for efficacy in a cell-based system with suitable controls. For example, to eliminate the possibility that non-antisense mechanisms are operating, the controls will consist of both randomized variants of the successful sequence or sequences containing the same residues and the corresponding sense sequences.

Example 2

Identification of oligonucleotides with optimum antisense activity against TNF α mRNA

Chimaeric oligonucleotide libraries were incubated with a target mRNA, in this case that of the TNF α gene.

Experimental methods:

Construction of Antisense Oligonucleotide Libraries:

To determine the optimal length of antisense oligonucleotides required for specific mRNA hybridisation, oligonucleotide libraries of 8 nt and 12nt have been synthesised by Oswel DNA Services Ltd. The libraries comprise of:

- Random nucleotide sequences comprising all possible combinations of sequences
- Specific anti-mRNA sequences to:
 - Series 1:** the 5' untranslated cap region (nucleotides 1 to 96 of mRNA, see Appendix 1)
 - Series 2:** a stable stem loop region within the mRNA transcript (nucleotides 1225 to 1321 of mRNA, see Appendix 2)
 - Series 3:** the 3' untranslated region (nucleotides 1489 to

1585 of mRNA, see Appendix 3)

Production of in vitro transcription construct:

TNF α cDNA was amplified from pE4 (a TNF α cDNA construct obtained from ATTC, American Type Culture Collection), using the following dUTP containing primers:

F1 (Sense) 5' -CAUCAUCAUTTCTGCTCTAAAAGCTGCTG-3'

R2 (Antisense) 5' -CUACUACUACUACCTAAGCAACCTTTATTTCTCG-3'

The dUTP tags provide both a means of directional cloning of the cDNA into the vector insertion pAMP1, and also play a crucial part in the cloning reactions themselves.

Optimal thermal cycling parameters were as follows:

Temperature	No. of Cycles	Time
96 C	1	2 mins
94 C	25	30 secs
53 C	1	30 secs
72 C	1	90 secs
72 C	1	10 mins

The amplified cDNA product was purified using the Quiaquick PCR purification kit (Quiagen). Once purified, 50 ng of cDNA was cloned into pAMP1 using the CloneAmp pAMP1 system for rapid cloning of amplification products (GibcoBRL) to create the plasmid pTNF8 according to the manufacturers protocol. The sequence integrity of the construct was confirmed by sequences analysis.

An in vitro transcript template was prepared by linearisation of 50 μ g pTNF8 by BamHI prior to transcription using the Ribomax large scale RNA production system - T7 RNA polymerase from Promega.

Unincorporated ribonucleotides and premature termination

transcripts were removed by spin column chromatography (Chromspin-400 DEPC treated, Clontech) and analysed by formaldehyde gel electrophoresis (Sambrook et al, 1989).

Cleavage of mRNA using gene specific libraries:

- 1) The following reaction was set up in a total volume of 100 μ l:
 - 20 μ M TNF α mRNA
 - 200 nM of each antisense TNF α oligonucleotide (12 nt or 8 nt of contiguous sequence)
 - 20 mM KCl
 - 10 mM MgCl₂
 - 20 mM Tris-HCl, pH 7.5
 - 0.1 mM EDTA
 - 0.1 mM DTT
- 2) The reaction was incubated at 37 °C for 5 minutes prior to the addition of 0.1 μ g / μ l of RNase H.
- 3) The above was incubated at 37 °C for 40 minutes.
- 4) RNase H activity was inhibited by phenol/chloroform extraction.
- 5) 20 ng glycogen was added to the reaction products.
- 6) Antisense oligonucleotide libraries were removed by spin column chromatography (Chromspin-30 DEPC treated, Clontech).
- 7) RNA was precipitated by the addition of 2.5 volumes (100 μ l) ethanol, 0.1 vols. 3M sodium acetate at pH 5.2.
- 8) Pelleted RNA was washed with 70 % ethanol, before resuspension in 16 μ l of DEPC treated water.

Cleavage of mRNA using random libraries:

The reaction protocol was as above for the gene specific libraries but using 20 μ M of the random library as supposed to 200 nM used above.

Primer Extension Analysis of mRNA fragments:

The oligonucleotide libraries were incubated with the in vitro transcribed TNF α mRNA and with RNase H. To identify the sequences flanking the cut sites, the fragments generated in the incubation above were amplified by PCR using several primers

targeted to various regions of the RNA thus ensuring that no combinations of cut fragments would be missed in the amplification steps.

Primer extension analysis experiments were performed, each using 2 pmol of one of the FAM-labeled primers listed below. This was used to prime the reverse transcription of 8 μ l of the RNA cleavage products, generated by the protocols above, using the Superscript II RNase H reverse transcriptase (GibcoRL) according to the manufacturers instructions. Primer extension reactions were performed in duplicate, as were RNase H negative controls for each primer tested.

The cDNA fragments so formed were analysed upon a 4 % acrylamide denaturing gel run in an ABI (Applied Biosystems Inc) 377 prism sequencing apparatus, and subsequent analysis using the GeneScan version 2.0 software.

Samples for the ABI 377 apparatus are fluorescently labelled, and gel separation is measured as the time taken for a band to reach a fluorescent detector which scans the gel at a specific point. The GeneScan software converts this time resolved data back to the usual format or can calculate sizes directly if fluorescently labelled size standards are present in the sample. Size standards will carry a different fluorescent marker to the samples.

Primers used in primer extension analysis:

Primer Code	Primer Sequence
F2	GATTCAGGAATGTGTGGCCT 956:CCTGG GATTCAGGAATGTGTGGCCT GCACA:975
F3	CAACCTCCTCCTCTGCCATCAAG 586:AAGCT CAACCTCCTCCTCTGCCATCAAG AGCCC:607
T8836	G TTCCTCAGCCTCTTCTCCTTC 178: TGCTT GTTCCTCAGCCTCTTCTCCTTC CTGAT:199
T8837	GAAAACGGAGGCTGAACAATAG 1366:TCCGT GAAAACGGAGGCTGAACAATAG GCTGT:1387
T8838	CTACTATTCAGTGGCCGAGAAA 1544:TCGGC CTACTATTCAGTGGCCGAGAAA TAAAG:1564

Principle of Quantative RNA PCR

Having determined the cut sites, it would be advantageous to determine which of the corresponding ODNs mediate cleavage most efficiently. To this end a quantitative RT PCR assay has been developed.

The quantity of uncleaved mRNA is assayed by analysing separate overlapping regions of the target mRNA using PCR reactions such that the whole mRNA is covered by the overlapping regions. With this approach the worker does not need to know the binding site of the antisense ODN and therefore this method is ideal for use in the random library approach as well as for targeted antisense

ODNs. Copies of the sequences corresponding to the overlapping regions that have been chosen are generated, with a reasonably sized specific internal deletion, e.g. about 50 bp, removed, such that they can be cloned to allow in vitro transcription of the deletion fragments. These deletion fragments can then be used as an internal standard RNA control for quantitative RT PCR.

A known amount of one of the standards is added to the RNA sample to be assayed. Following reverse transcription of this mixture, using a gene specific primer, PCR is carried out using fluorescently labeled primers and the reaction is run on an ABI 377 prism sequencing apparatus and the peaks corresponding to the amount of each product, is determined. Since the starting quantity of the standard is known, the amount of other products can be deduced by calibrating the intensity of the fluorescence of the products against that of the standard.

Producing an internal standard like this overcomes many of the problems associated with quantitative RT PCR. Firstly, it acts to control for varying efficiencies between individual RT reactions and secondly, as the standard and the PCR target have the same primer sequence, and the same internal sequence (apart from the internal deletion of the control) they will behave in a PCR reaction in the same way and should thus amplify with equal efficiency when the PCR reaction is stopped in the log phase.

Methodology of Quantitative RNA PCR

The TNF α mRNA was divided into three overlapping PCR fragments for analysis, these being fragment A (bases 14 - 557), fragment B (bases 502 - 1062) and fragment C (bases 1035 - 1575).

Primer sequences :	A	forward GCTGCCAGGCAGGTTCTCTTCC reverse CTGATGGTGTGGGTGAGGAGC
	B	forward CTTCAAGGGCCAAGGCTGCCC reverse GCTCCCTGGTCTCCAGATTCC
	C	forward GCCTACAGCTTTGATCCCTG reverse GCAACCTTTATTTCTCGCCACTG

(all forward primers are labelled with a 5' FAM fluorescent moiety)

1. Production of Internal Competitive RNA Standards

In order to obtain fragments A,B and C with a 50bp deletion (approx.), for use as internal competitive RNA standards, 'deletion primers' were produced. These primers have the appropriate forward sequence (either A, B or C) followed by 20bp (approx.) of sequence 50bp(approx.) 3' to the forward sequence. When PCR is performed with deletion primers and the appropriate reverse primers the fragments generated are effectively the A,B or C fragments with a 50bp (approx.) deletion 3' to the forward primer.

Fragment A deletion forward primer

GCTGCCAGGCAGGTTCTCTTCCGCACTGAAAGCATGATCCGGG

Fragment B deletion forward primer

CTTCAAGGGCCAAGGCTGCCCCAAGGTCAACCTCCTCTCTGC

Fragment C deletion forward primer

GCCTACAGCTTTGATCCCTGGGACTTGAGAAGACCTCACC

1.1 PCR reactions were performed on a Perkin Elmer 9600 PCR machine using Perkin Elmer Amplitaq polymerase (1 unit) with the manufactures buffer with 30pmole of each of the appropriate forward deletion primer and reverse primer; 1.5mM MgCl₂; 10mM dNTP's; 10ng of pJNF8 TNF α clone. Cycling times were 1 cycle of 95°C 5 min followed by 35 cycles of 95°C for 30 sec, 48°C for 1 min, 72°C for 2 min with a single final cycle of 72°C for 10 min.

1.2 PCR products were separated on a 2% agarose gel. Bands were excised and purified with the Qiagen QIAquick Gel Extraction Kit according to the manufactures protocol. Purified bands were cloned into the pCR 2.1 vector (Invitrogen) using the TA Cloning Kit (Invitrogen) according to the manufactures protocol. Sequence and orientation of the inserts was checked by sequencing using standard methods.

1.3 Clones which yield RNA with the same sense as TNF α mRNA, when transcribed from the T7 RNA polymerase site in pCR2.1, were selected. RNA for each deletion fragment was transcribed from the appropriate clones using the RiboMAX RNA Production System with T7 RNA polymerase (Promega) according to manufactures instructions. The transcribed RNA was then purified on a Sephacryl S-500 HR gel filtration column (Pharmacia) using a DEPC treated 25mM Tris-HCl, 150mM NaCl, 24mM MgCl₂ pH7.4 buffer under gravity flow at 4°C. RNA was eluted from the column at 110-130ml of flow through and was precipitated using 2.5 volumes of 100% ethanol and resuspended in 100uL distilled water.

The RNA TNF α deletions (internal competitive RNA standards) will now be referred to as AD (for the A fragment deletion), BD (for the B fragment deletion) and CD (for the C fragment deletion).

2. Quantification of Cleaved TNF α mRNA by Competitive RNA PCR

To describe this methodology a specific example is used:

An antisense cleavage experiment is performed using an anti sense oligo which cleaves within the C fragment. This cleaved RNA will from now on be referred to as X.

2.1 Six EZ rTth RNA PCR (Perkin Elmer) reactions are set up according to the manufactures instructions each containing 0.05ng of X with primers for fragment C (numbered C1-C6) and six identical reactions with primers for fragment B (numbered B1-B6). A serial dilution of CD is then added to C1-6, ie 50ng of CD to C1; 5ng of CD to C2; 0.5ng of CD to C3; 0.05ng of CD to C4; 0.005ng of CD to C5; 0.0005ng of CD to C6. A corresponding serial dilution of BD is also added to B1-6.

2.2 The RNA PCR reaction is then performed on a Perkin Elmer 9600 PCR machine using the following cycles. 1 cycle of 94°C for 0 sec; 48°C for 0 sec; ramp to 65°C over 5 min and hold for 35 min, 1 cycle of 95°C for 1min, 32 cycles* of 95°C for 25 sec; 65°C for 1 min, 1 cycle 65°C for 7 min.

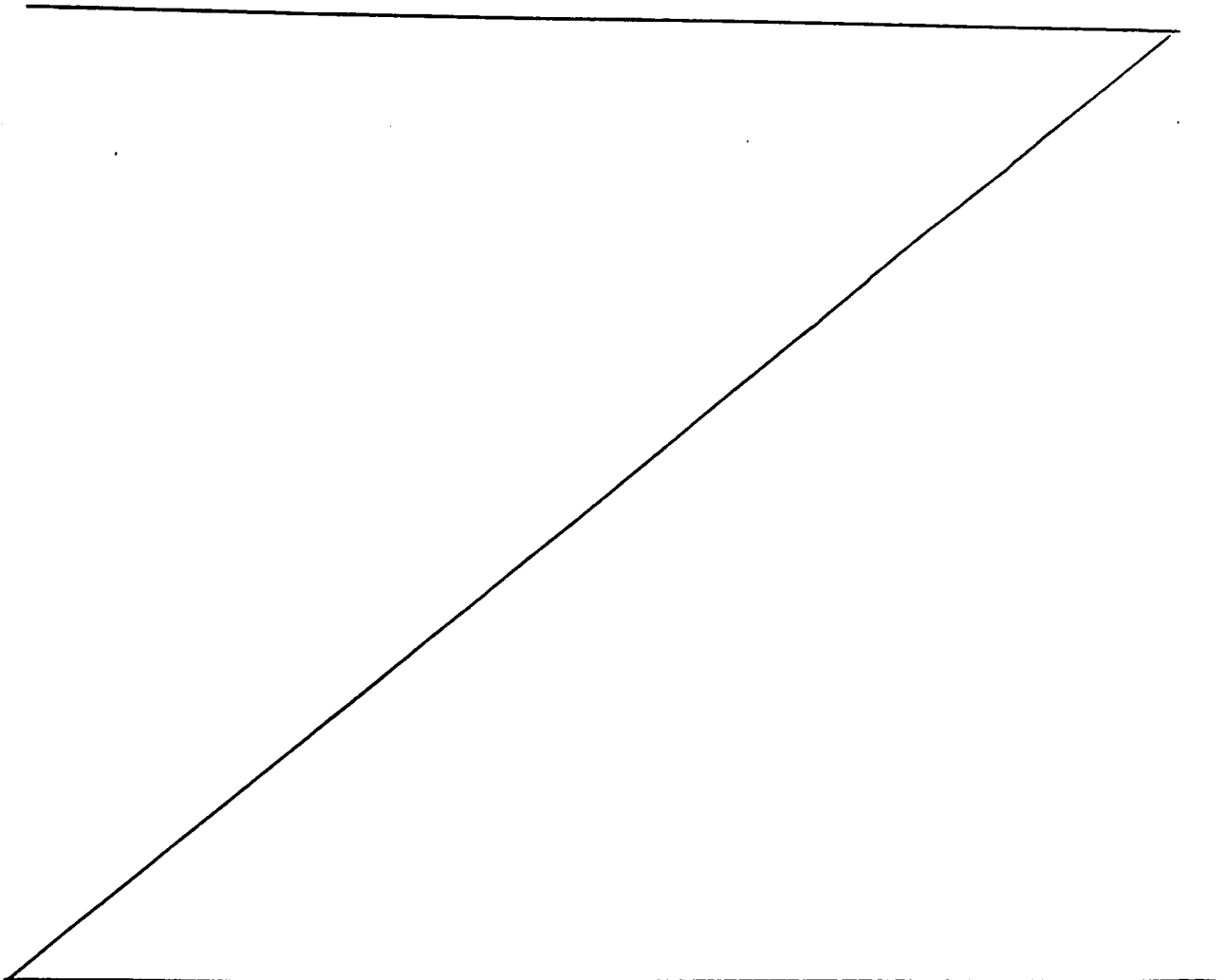
(*32 cycles are used so that the reaction stops while still in log phase)

2.3 The reactions are then run on an ABI 377 and the peak heights of the C/B fragments and CD/BD fragments collected for each sample.

2.4 A graph is then plotted of CD concentration against C and CD peak height (see Fig 7a), and similar Figure 7b plotted for the B reactions. The point at which the two lines

cross refers to the point at which the concentration of CD RNA is equimolar to the concentration of available C fragment RNA. As the antisense oligo cleaves within fragment C the concentration of available C RNA will be lower than the concentration obtained at the cross over point from the B reaction graph.

Hence, by comparing this two figures it is possible to quantify the cleavage of $\text{TNF}\alpha$ by the antisense oligo.



Results:Primer extension of fragments from series 1 of 12 nt gene specific oligonucleotides:

The primer used for primer extension experiments was T8836.

Fragments of 124 nt and 134 nt were detected. These correspond to antisense cut sites in the mRNA at nucleotides 76 and 66. (See figure 8)

Cleavage site 76 corresponds to antisense oligonucleotide:
GTGTCCTTTCCA

Cleavage site 66 corresponds to antisense oligonucleotide:
GGGGAGAGAGGG

Primer extension of fragments from series 1 of 8 nt gene specific oligonucleotides:

The primer used for primer extension experiments was T8836.

Fragments of 121 nt and 136 nt were detected. These correspond to antisense cut sites in the mRNA at nucleotides 79 and 64. (See figure 9)

Cleavage site 79 corresponds to antisense oligonucleotide:
CCTTTCCA

Cleavage site 64 corresponds to antisense oligonucleotide:
GGGGAGAG

Primer extension of fragments from series 2 of 12 nt gene specific oligonucleotides:

The primer used for primer extension experiments was T8838.

Major fragments of 261 nt, 265 nt and 275 nt were detected. These correspond to antisense cut sites in the mRNA at nucleotides 1289, 1297 and 1303. (See figure 10)
Some minor peaks were detected in earlier experiments, under

slightly differing conditions, corresponding to fragments at 281 and 282 nt.

Cleavage site 1303 corresponds to antisense oligonucleotide:
GGTCTCCCAAAT

Cleavage site 1297 corresponds to antisense oligonucleotide:
AAATACATTCAT

Primer extension of fragments from series 2 of 8 nt gene specific oligonucleotides:

The primer used for primer extension experiments was T8838.

The major fragment was detected at 264 nt. These correspond to antisense cut sites in the mRNA at nucleotides 1300. (See figure 11)

Cleavage site 1300 corresponds to antisense oligonucleotide:
TCCCAAAT

Primer extension of fragments from series 3 of 12 nt gene specific oligonucleotides:

The primer used for primer extension experiments was T8838.

Unfortunately, no reliable data was acquired from these experiments or for series 3 8mer libraries.

Primer extension of fragments from random 12 nt oligonucleotides:

All five of the primers, listed in the method, were used in these primer extension experiments:

Primer	Fragment Lengths	Cut Sites
F2	112	863 nt
F3	100	507 nt
T8836	69	131 nt
T8837	None	None
T8838	104	1460 nt

Site of mRNA cleavage	Predicted Antisense Sequence
-----------------------	---------------------------------

131 nt	GAGCGCCTCCTC
507 nt	TGGCCCTTGAGA
1460 nt	TAGACAACTTAA

Results of quantitative RNA PCR on series 2 oligonucleotides:

The results of these experiments, under the conditions used, did not detect any difference in the cleavage efficiency of the oligonucleotides tested. The results suggested that all 5 cleaved with almost 100% efficiency.

oligo	b peak height	b d peak height (@5ng con)	amount b (5xb peak height/bd peak height)	c peak height	c d peak height (@5ng con)	amount c (5xb peak height/cd peak height)
TCATCTGT	130680	16906	38.6	75712	139347	2.7
AAATACAT	73597	42672	8.6	0	96686	0
TCCCAAAT	108621	34437	15.7	22336	1104770	1.06
AAATACATT	na	na	na	49785	234045	1.06
CAT						
GGTCTCCCA	81504	51354	7.9	0	40134	0
AAT						

Oligonucleotides tested:

1283-90	TCATCTGT
1291-98	AAATACAT
1299-1306	TCCCAAAT
1287-98	AAATACATTTCAT
1299-1310	GGTCTCCCAAAT

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Appendix

T.N.F. Alpha Oligonucleotides.SENSE Oligonucleotides.SERIES 1.

(1-96 bp)

8-oligonucleotides.

- 1.) CACACCCT.
- 2.) GACAAGCT.
- 3.) GCCAGGCA.
- 4.) GGTTCTCT.
- 5.) TCCTCTCA.
- 6.) CATACTGA.
- 7.) CCCACGGC.
- 8.) TCCACCCT.
- 9.) CTCTCCCC.
- 10.) TGGAAAGG.
- 11.) ACACCATG.
- 12.) AGCACTGA.

12-Oligonucleotides.

- 1.) CACACCCTGACA.
- 2.) AGCTGCCAGGCA.
- 3.) GGTTCTCTTCCT.
- 4.) CTCACATACTGA.
- 5.) CCCACGGCTCCA.
- 6.) CCCTCTCTCCCC.
- 7.) TGGAAAGGACAC.
- 8.) CATGAGCACTGA.

SERIES 2

(1225-1321 bp.)

8-Oligonucleotides.

- 1.) CTCTATTT.
- 2.) ATGTTTGC.
- 3.) ACTTGTGA.
- 4.) TTATTTAT.
- 5.) TATTTATT.
- 6.) TATTATTT.
- 7.) ATTTATTT.
- 8.) ACAGATGA.
- 9.) ATGTATTT.
- 10.) ATTTGGGA.
- 11.) GACCGGGG.
- 12.) TATCCTGG.

12-Oligonucleotides.

- 1.) CTCTATTTATGT.
- 2.) TTGCACTTGTGA.
- 3.) TTATTTATTATT.
- 4.) TATTTATTATTT.
- 5.) ATTTATTTACAG.
- 6.) ATGAATGTATTT.
- 7.) ATTTGGGAGACC.
- 8.) GGGGTATCCTGG.

SERIES 3

(1489-1585 bp)

8-Oligonucleotides.

- 1.) CTGTCACT.
- 2.) CATTGCTG.
- 3.) AGGCCTCT.
- 4.) GCTCCCCA.
- 5.) GGGAGTTG.
- 6.) TGTCTGTA.
- 7.) ATCGGCCT.
- 8.) ACTATTCA.
- 9.) GTGGCGAG.
- 10.) AAATAAAG.
- 11.) GTTGCTTA.
- 12.) GGAAAGAA.

12-Oligonucleotides.

- 1.) CTGTCACTCATT.
- 2.) GCTGAGGCCTCT.
- 3.) GCTCCCCAGGGA.
- 4.) GTTGTGTCTGTA.
- 5.) ATCGGCCTACTA.
- 6.) TTCAGTGGCGAG.
- 7.) AAATAAAGGTTG.
- 8.) CTTAGGAAAGAA.

CLAIMS:

1. A chimaeric oligonucleotide library for use in identifying an antisense binding site in a target mRNA, which comprises a plurality of distinct chimaeric oligonucleotides capable of hybridizing to mRNA to form a duplex, the nucleotide sequences of which each have a common length of 7 to 20 bases and are generated randomly or generated from information characterising the sequence of the target mRNA, wherein substantially all the nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library, and wherein each nucleotide sequence comprises:

a) a recognition region comprising a sequence of nucleotides which is recognisable by a duplex-cutting RNAase when hybridized to the mRNA, and

b) a flanking region comprising a sequence of chemically-modified nucleotides which binds to the mRNA sufficiently tightly to stabilise the duplex for cutting of the mRNA in the duplex by the duplex-cutting RNAase, wherein the nucleotides constituting the flanking region are different from those constituting the recognition region, and wherein each oligonucleotide is protected against exonuclease attack.

2. A library according to claim 1, wherein the common length of the nucleotide sequences is 10 to 20 bases.

3. A library according to claim 1 or claim 2, wherein the recognition region comprises a sequence of nucleotides which is recognisable by RNAase H when hybridized to the mRNA.

4. A library according to any one of claims 1 to 3, wherein the nucleotides constituting the recognition region are deoxyribonucleotides or phosphorothioate deoxyribonucleotides.

5. A library according to any one of the preceding claims,

wherein the recognition region comprises at least four nucleotides.

6. A library according to claim 5, wherein the recognition region comprises 5 to 10 nucleotides.

7. A library according to any one of the preceding claims, wherein the chemically modified nucleotides constituting the flanking region are 2' modified in the sugar moiety.

8. A library according to claim 7, wherein the chemically-modified nucleotides are 2'-O methyl ribonucleotides or 2'-O allyl ribonucleotides.

9. A library according to any one of claims 1 to 6, wherein the chemically-modified nucleotides constituting the flanking region are modified in the base moiety and are selected from 5-propynyl deoxyuridine, 5-propynyl deoxycytidine and 2-amino adenine analogues.

10. A library according to any one of claims 1 to 6, wherein the chemically-modified nucleotides constituting the flanking region are modified in the phosphate moiety and are selected from methyl phosphonate, triester, phosphoramidate, phosphorodithirite and boranophosphate analogues.

11. A library according to any one of the preceding claims, wherein the nucleotide sequences each comprise two flanking regions, one on either side of the recognition region.

12. A library according to claim 11, wherein each of the flanking regions is protected against exonuclease attack.

13. A library according to claim 12 wherein each of the flanking regions is protected by reverse T.

14. A library according to any one of the preceding claims, which is generated randomly by means of an oligonucleotide sequence synthesizer and comprises at least 10^6 distinct nucleotide sequences.

15. A library according to any one of claims 1 to 13, which is generated from information characterising the sequence of a target mRNA of known sequence by programming an oligonucleotide sequence synthesizer with the information so as to generate distinct sub-sequences of common length.

16. Use of an oligonucleotide library in a method of identifying an antisense binding site in a target mRNA, wherein the oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases and each of which comprises a substrate for a duplex-cutting RNAase if hybridised to the mRNA, which library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library.

17. Use according to claim 16, wherein the common length of the nucleotide sequences is 10 to 20 bases.

18. Use according to claim 16 or claim 17, wherein each nucleotide sequence comprises a phosphorothioate deoxyribonucleotide sequence.

19. Use according to any one of claims 16 to 18, wherein the oligonucleotide library is as defined in any one of claims 1 to 15.

20. A method of identifying an antisense binding site in a target mRNA, which comprises:

- 1) incubating with the target mRNA an oligonucleotide

library and a duplex-cutting RNAase under conditions to produce target mRNA cut at the antisense binding site; and

2) identifying the antisense binding site from the position of the cut in the mRNA; wherein the oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases and each of which comprises a substrate for the duplex cutting RNAase if hybridized to the mRNA; and wherein the oligonucleotide library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of such common length which are present as sub-sequences in the target mRNA are present in the library.

21. A method according to claim 20, wherein the oligonucleotide library is as defined in any one of claims 1 to 15.

22. A method according to claim 20 or claim 21, wherein the duplex-cutting RNAase is from a cell extract.

23. A method according to claim 22, wherein the cell extract also contains the target mRNA.

24. A method according to any one of claims 20 to 23, wherein the position of the cut in the mRNA is determined by sequencing isolated cut target mRNA.

25. A method according to claim 24, wherein the cut target mRNA is amplified prior to isolation.

26. A method according to claim 25, wherein the cut target mRNA is amplified by reverse transcription and polymerase chain reaction.

27. A method of producing an antisense oligonucleotide, which method comprises identifying an antisense binding site

according to any one of claims 20 to 26, and synthesizing an oligonucleotide capable of binding to the site.

Fig.1.

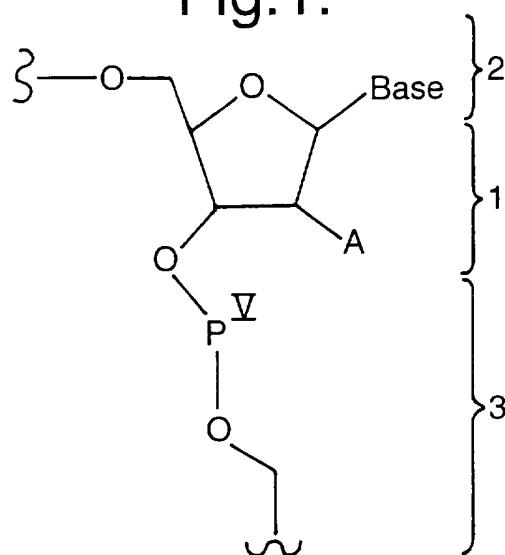
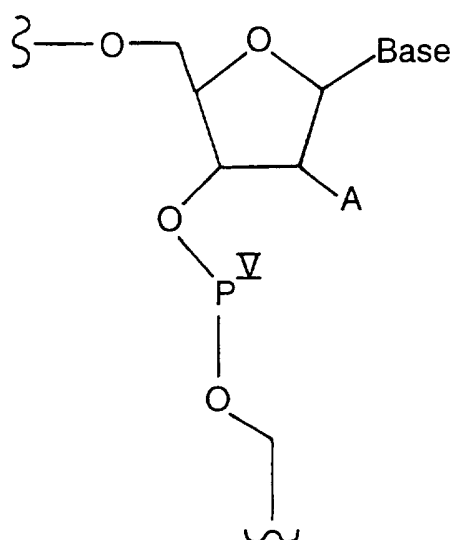


Fig.2.



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Fig.3.

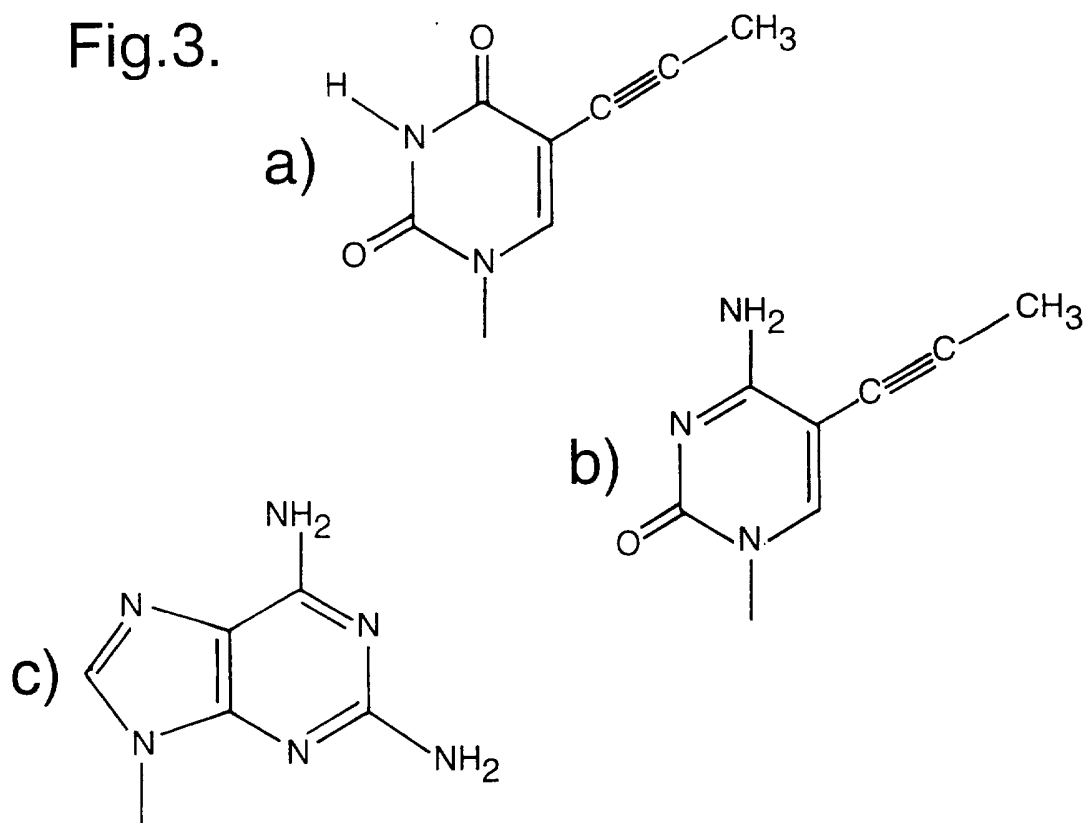


Fig.4.

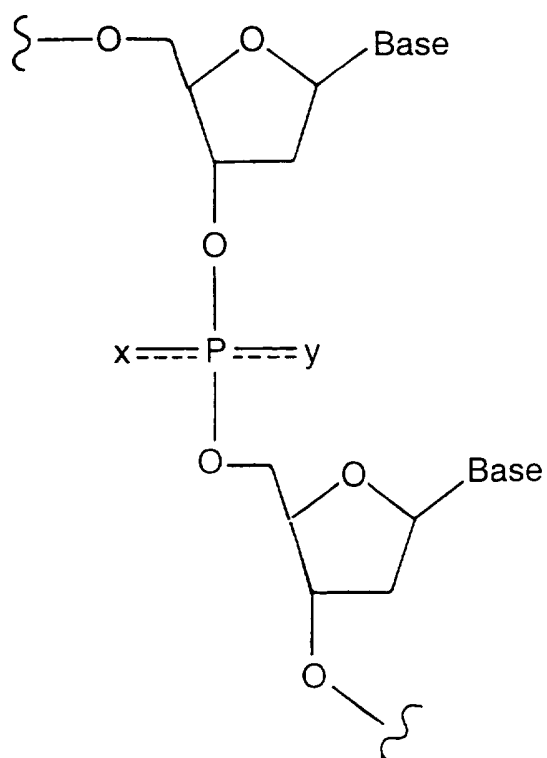
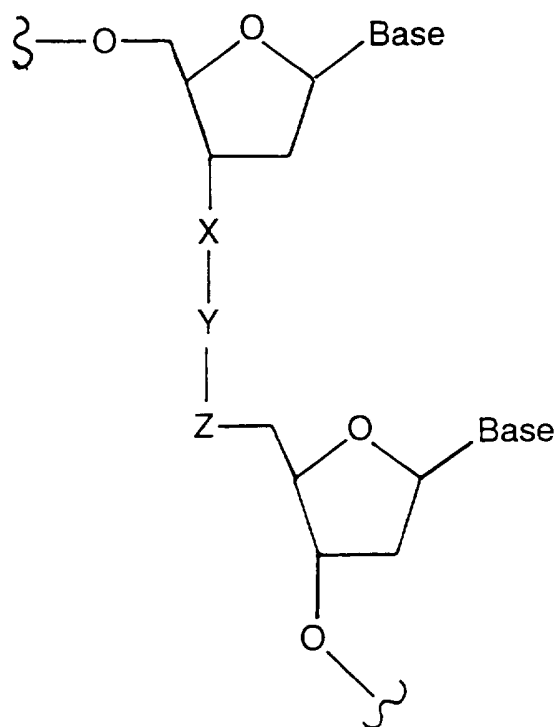
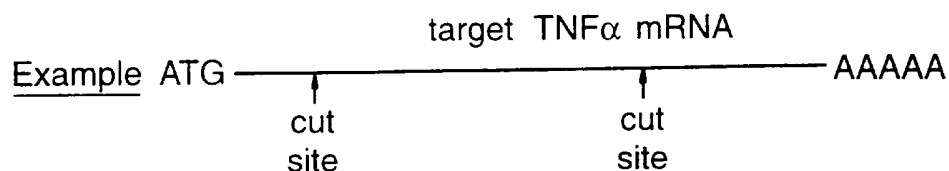


Fig.5.



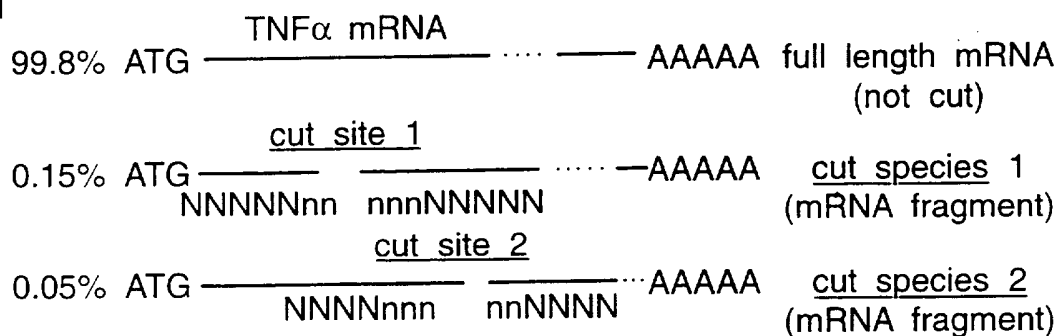
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Fig.6.

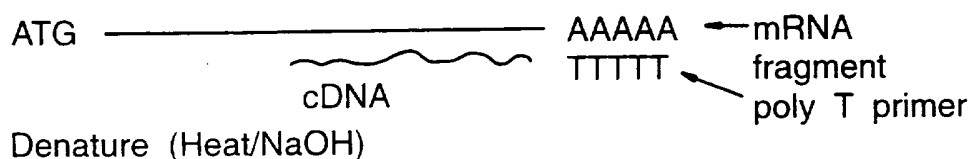


1. CAO_S NNNNNnnnnnnNNNNN :(15-mer)

Yield



2. RT-PCR synthesis of 1st cDNA strand:



- 3.

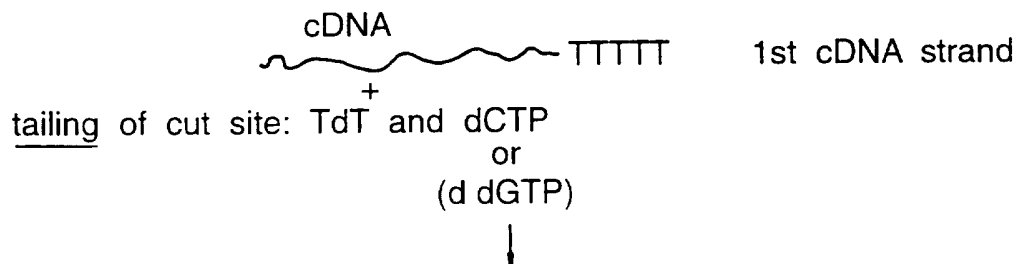
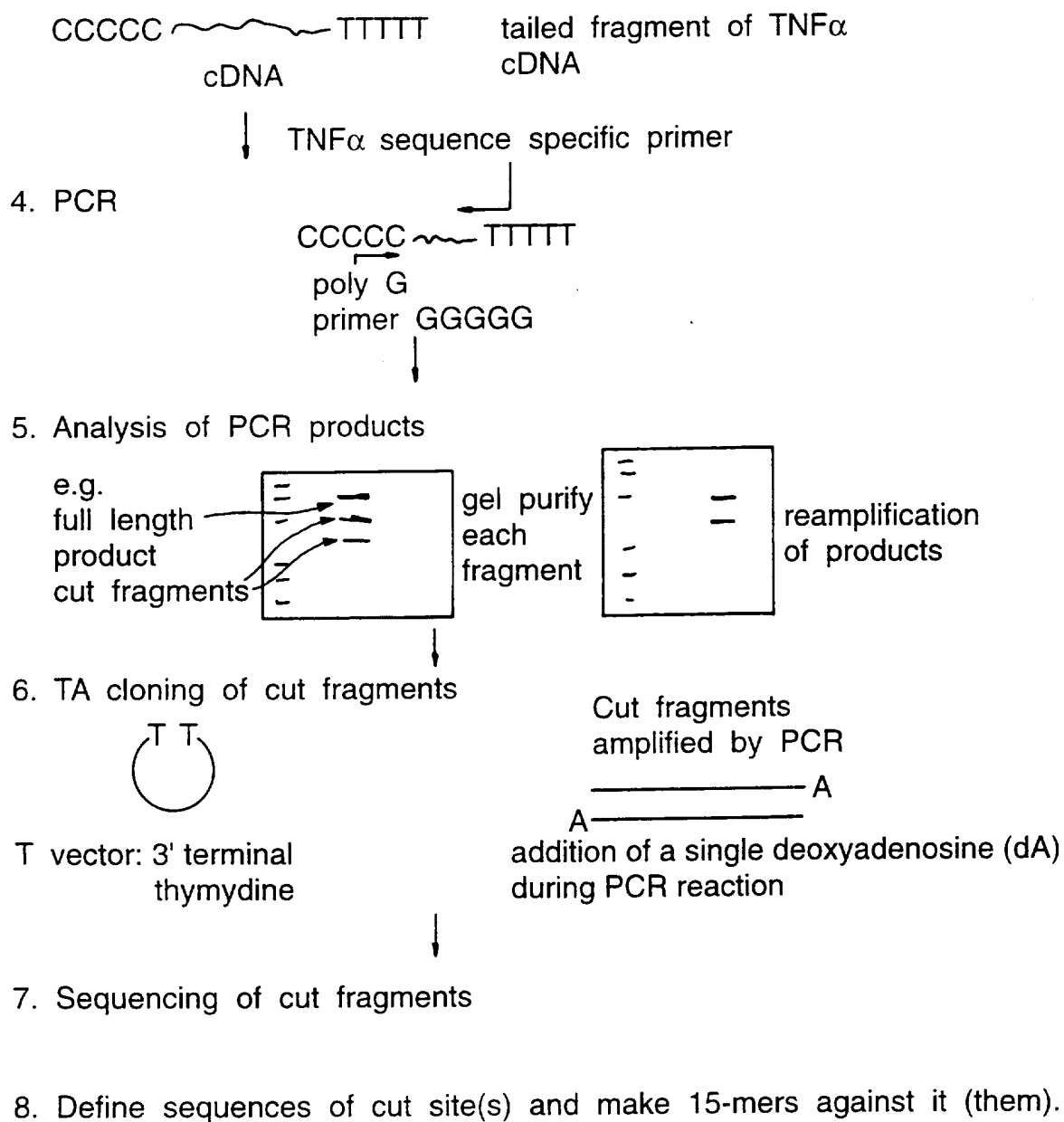


Fig.6 (Cont).



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Fig.7a.

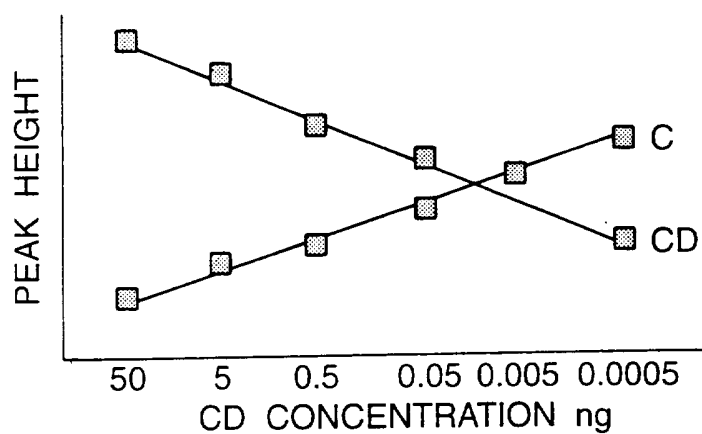
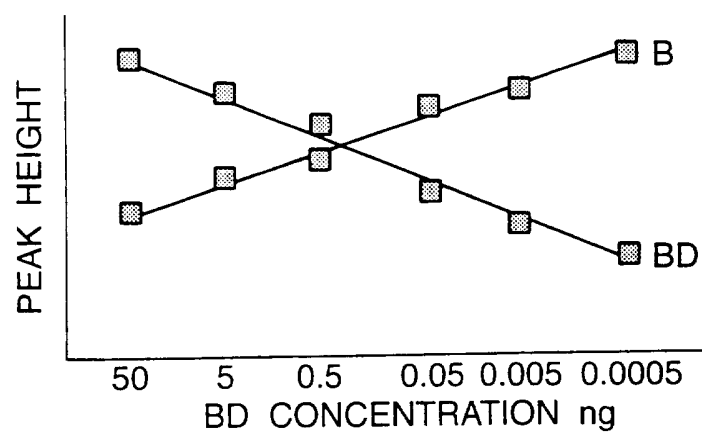
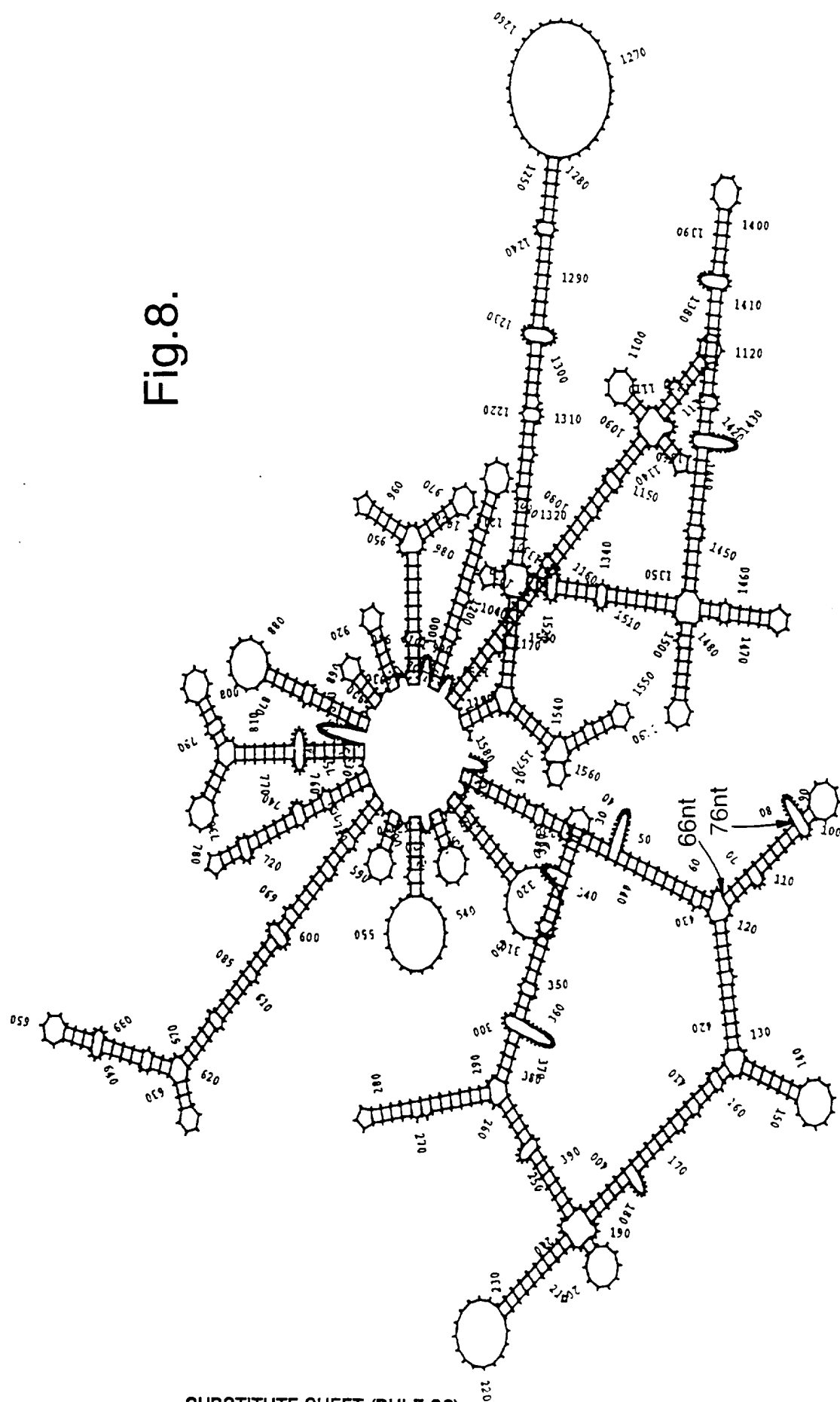


Fig.7b.



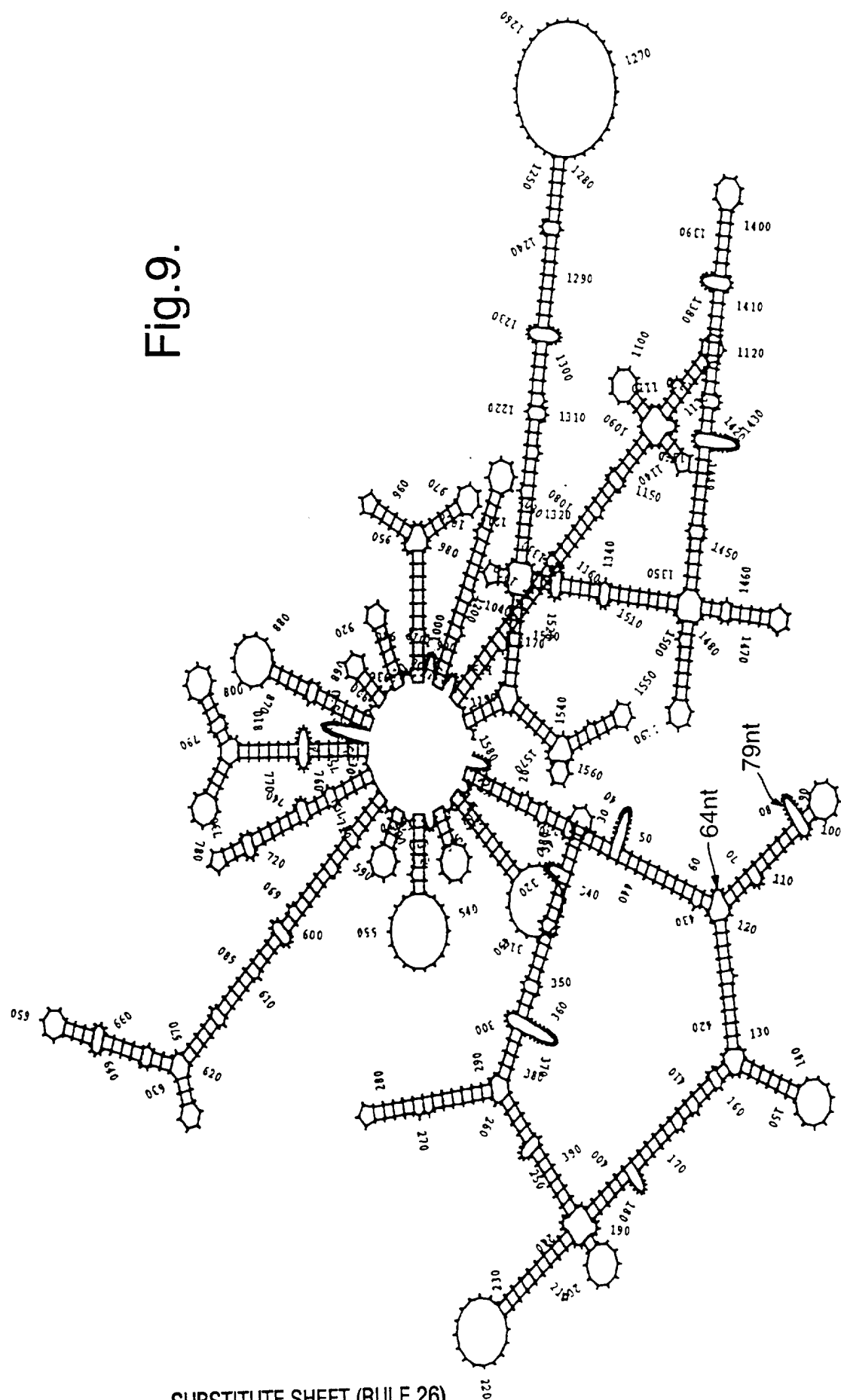
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Fig.8.



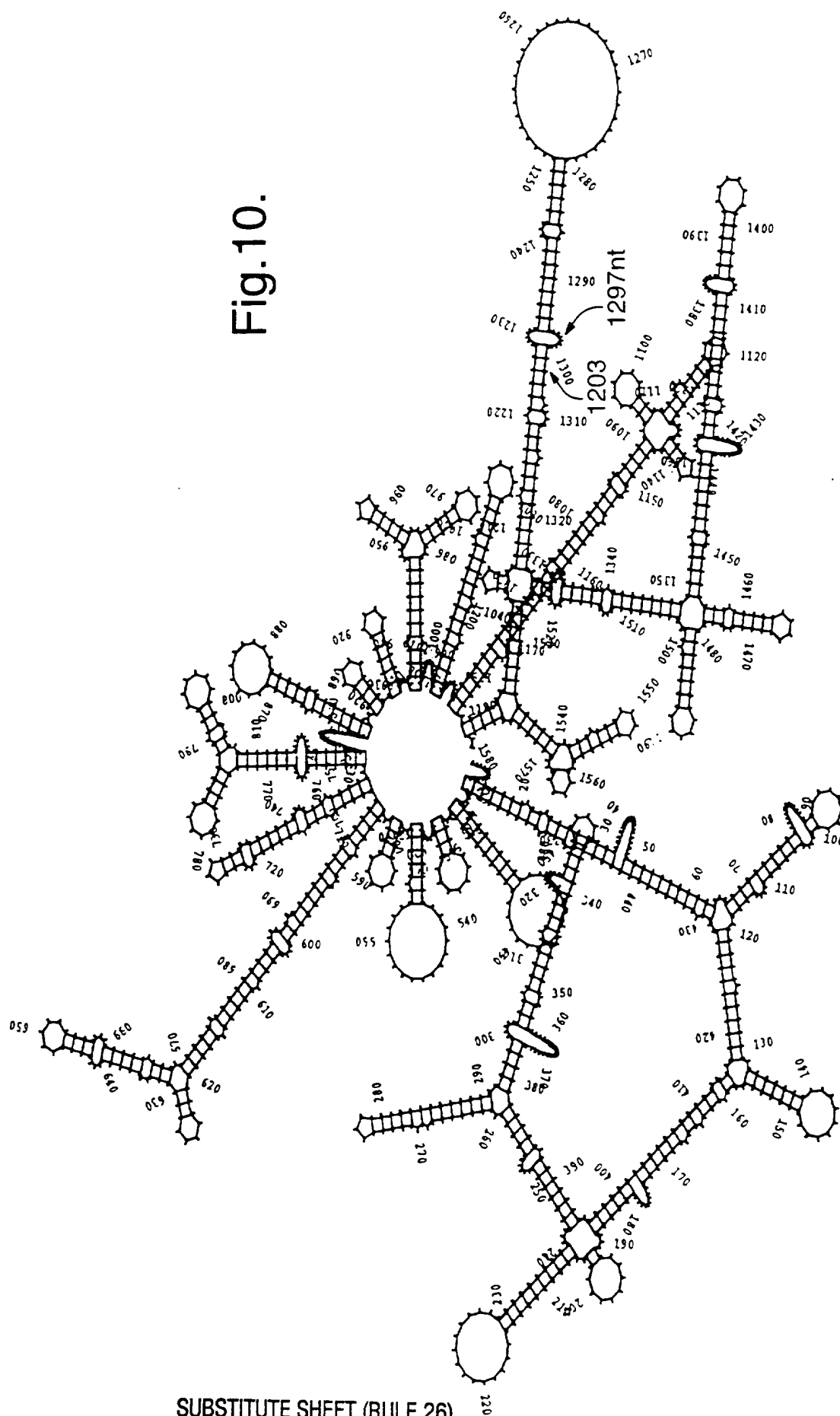
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Fig.9.



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Fig.10.



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Fig. 12.

