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(54) Title: RNASE L ACTIVATORS AND ANTISENSE OLIGONUCLEOTIDES EFFECTIVE TO TREAT RSV INFECTIONS

(57) Abstract

The invention concerns compounds and methods for treating infection with Respiratory Syncytial Virus. The compounds comprise an antisense portion, which is complementary to a normally single stranded portion of the RSV antigenomic strand (the mRNA strand), a linker and an oligonucleotide activator of RNase L, a ubiquitous non-specific RNase. The method comprises forming a complex of an activated RNase L and the antisense molecule. The application teaches methods of determining which portions of the RSV antigenomic strand are normally single-stranded. The application teaches that an antisense oligonucleotide having the sequence of residues 8281-8299 of the RSV genome is particularly useful to practice the invention and provides *in vitro* results superior to those obtainable with the conventional drug of choice, ribavirin.

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RNase L Activators and Antisense Oligonucleotides Effective to Treat RSV Infections

This application claims benefit of U.S. provisional application Serial No. 60/011,725, filed February 15, 1996.

1. FIELD OF THE INVENTION

The present invention concerns compounds useful for treating humans infected by Respiratory Syncytial Virus, a negative strand RNA virus, and methods of their use. Particularly, the invention concerns a complex of an oligonucleotide that is complementary to some portion of the anti-genomic strand of RSV and a covalently linked activator of RNase L (henceforth, "activator-antisense complexes"). More particularly, the invention concerns activator-antisense complexes, in which the oligonucleotide is selected to bind to a portion of the RSV anti-genomic strand that normally has no self-hybridizing secondary structure.

2. BACKGROUND TO THE INVENTION

Respiratory syncytial virus (RSV), a non-segmented, negative-strand RNA virus in the pneumovirus subfamily of *Paramyxoviridae*, is a widespread human pathogen accounting for over 1 million deaths per year worldwide (McIntosh and Chanock 1990). While the majority of serious cases are children from developing countries, there are estimated to be 300,000 hospitalized cases per year in the United States (Zisson, 1993). It is also believed that of childhood deaths from pneumonia caused by respiratory viral infections, 62% are due to RSV (Heilman, 1994). The only approved treatment for RSV is aerosolized ribavirin (1-b-D-ribofuranosyl-1,2,3-triazole-3-carboxamide). Ribavirin is administered as an aerosol which is inhaled. Ribavirin therapy has several limitations including minimal efficacy in clinical use, the requirement of a tent around the patient, the potential to clog ventilating units, and the observation of some teratogenicity in animal models (Froelich, 1994), significant side effects and high cost.

RSV replicates in several alveolar cell types including macrophage and epithelial lineages (Panuska et al., 1992,

Midulla et al., 1993). Accordingly, ribavirin is administered to RSV infected individuals by inhalation of an aerosol. Taber et al., 1983, Pediatrics 72:613-18; Hall et al., 1983, N. Eng. J. Med. 308:1443-7; Englund et al., 1994, J. Pediatrics 125:635-41.

Activator-antisense complexes (termed therein "2-5A:AS") have been described previously (Torrence et al., 1993, WO 94/09129 by Torrence et al.). Although antisense oligonucleotides have been used as antiviral agents, e.g.: to inhibit HIV replication, see Zamecnik et al, 1986; Goodchild et al., 1988; Letsinger et al., 1989; Balotta et al., 1993; to inhibit RSV infection, WO95/22553 by Kilkuskie et al., no examples of the successful use of activator-antisense complexes as an antiviral therapy have been reported.

The mechanism of action of activator-antisense complexes is different than the mechanism of action of other antisense oligonucleotides. The activator portion of the activator-antisense complexes activates RNase L and the antisense domain serves as a specific, high affinity binding site for the target RNA. The result is the selective cleavage of the target RNA by RNase L.

Physiologically, RNase L functions as part of the interferon system in restricting virus replication in cells of higher vertebrates (reviewed in Silverman, 1994). Interferon treatment of cells activates genes encoding 2-5A synthetases, double-stranded RNA (dsRNA)-dependent enzymes that produce 5'-triphosphorylated, 2',5'-linked oligoadenylates (2',5'A) from ATP. Viral dsRNAs are potential activators of these enzymes (Gribaudo et al., 1991). The 2',5'A binds to and activates RNase L resulting in the general cleavage of cellular and viral RNA; thus restricting the replication of some picornaviruses (Chebath et al., 1987; Rysiecki et al., 1989; and Hassel et al., 1994).

RNase L is not specific for cleaving viral RNA. For instance, in interferon-treated, encephalomyocarditis virus

infected cells, RNase L causes degradation of ribosomal RNA (Wreschner et al., 1981). Through the activator-antisense approach, RNase L is converted from a non-specific nuclease to a highly specific endoribonuclease that selectively cleaves mRNA targets. This has been demonstrated in a cell-free system from Daudi cells, a human lymphoblastoid cell line, in which a modified HIV-1 vif mRNA was targeted for cleavage by an activator-antisense complex (Torrence et al., 1993). Subsequently, purified RNase L has been directed by an activator-antisense complex to cleave selectively an mRNA target encoding the protein kinase PKR in the presence of a nontargeted mRNA (Maran et al., 1994). Furthermore, in HeLa cells, the use of activator-antisense complexes, which were directed to a sequence in PKR mRNA, resulted in the ablation of PKR mRNA and enzyme activity (Maran et al., 1994) such that the dsRNA-mediated activation of transcription factor, NF-kB was ablated. More recently, it was shown that the activation of RNase L by an activator-antisense complex results in the catalytic degradation of PKR mRNA (k_{cat} of about 7 sec^{-1}) (Maitra et al., 1995).

3. SUMMARY OF THE INVENTION

The present invention provides a complex that is useful for the treatment of infection by RSV. The essential components of the complex are an antisense oligonucleotide which has a sequence that is complementary to between about 10 and about 30 nucleotides of the antigenomic RNA strand, i.e., the template strand for genome synthesis, of a strain of RSV and an activator of RNase L (henceforth, "activator-antisense complexes"). The elements of the activator-antisense complex are preferably covalently linked by a linker.

In an alternative embodiment, the invention consists of a non-covalently linked complex comprising one or two activated RNase L molecules and at least one antisense oligonucleotide complementary to between about 10 and 30 nucleotides of the antigenomic RNA strand of RSV (henceforth,

"enzyme-antisense complexes"). In a further alternative embodiment the invention consists of an antisense oligonucleotide having a sequence of at least 10-30 nucleotides and preferably 15-25 nucleotides, and more preferably which is 18 or 19 nucleotides.

The activator-antisense complexes of the invention are transported across the cell membrane without the use of carriers or permeabilizing agents. Once internalized the activator-antisense complexes lead to the formation of enzyme-antisense complexes, which causes destruction of the antisense targeted RNA. To treat RSV infection the antisense complexes can be administered by inhalation of an aerosol, the same method as is used to administer ribavirin. Ribavirin and the antisense complexes of the invention can, therefore, be administered in a common pharmaceutical composition.

4. BRIEF DESCRIPTION OF THE FIGURES

Figures 1:1-1:10. The sequence of Respiratory Syncytial Virus strain A2, positions numbered in the 5'→3' direction. Figures 2A-2H:3. Squiggle plot output of MFOLD calculations of the secondary structure of portions of the RSV antigenomic RNA, positions numbered in 5'→3' order. Figure 2A. Squiggle plot of residues 7900-8800 of RSV antigenomic RNA. Figures 2B:1-2B:3. Three alternative squiggle plots of residues 1-1124 of RSV antigenomic RNA. Figures 2C:1-2C:3. Three alternative squiggle plots of residues 1100-2400 of RSV. Figures 2D:1-2D:3. Three alternative squiggle plots of residues 2200-3300 of RSV antigenomic RNA. Figures 2E:1-2E:2. Two alternative squiggle plots of residues 3100-4300 of RSV antigenomic RNA. Figures 2F:1-2F:3. Three alternative squiggle plots of residues 4200-5599 of RSV. Figures 2G:1-2G:3. Three alternative squiggle plots of residues 5600-6999 of RSV antigenomic RNA. Figures 2H:1-2H:3. Three alternative squiggle plots of residues 6600-7999 of RSV antigenomic RNA.

Figure 3. Comparison of anti-RSV activities of spA_4-antiRSV3'-3'T/(8281-8299) and spA_2-antiRSV3'-3'T/(8281-8299).

5. DETAILED DESCRIPTION OF THE INVENTION

The invention in one embodiment consists of the covalently-linked complex of an activator of RNase L and an oligonucleotide that is capable of binding to the antigenomic template RNA strand of RSV and/or binding to an mRNA of an RSV protein (an "RSV antisense oligonucleotide"). In an alternative embodiment the invention consists of the non-covalently linked complex of an activated RNase L and an RSV antisense oligonucleotide.

In a preferred embodiment the antisense oligonucleotide is complementary to a portion of the RSV antigenome that is normally single stranded. The activator is attached through a linker to either the 3' or the 5' terminus of the antisense oligonucleotide by a linker. In one embodiment, a blocker is attached to the 3' terminus of antisense oligonucleotide and the linker is attached to the 5' terminus of the antisense oligonucleotide. In an alternative embodiment the linker is attached to the 3' end of the antisense oligonucleotide and serves as both linker and blocker. The antisense oligonucleotide is between about 15 and about 20 nucleotides in length and preferably 18 or 19 nucleotides in length. Those skilled in the art will understand that oligonucleotides with high GC content can be shorter than those with low GC content.

According to the invention, the portion of the antigenome of a strain of RSV to which the antisense oligonucleotide is complementary can be determined from the sequence of the strain of RSV and secondary structure determining algorithms such as MFOLD. A suitable portion of the RSV antigenome is one that is normally in a single stranded conformation, e.g., forms a loop of the stem and loop secondary structure of RNA.

Because RSV is a negative strand virus, the antisense oligonucleotides are complementary not only to the

antigenomic RNA but also to the mRNA that directs translation of the viral proteins.

The internucleotide phosphodiester bonds of the antisense oligonucleotide can be any bonds that are compatible with the formation of Watson-Crick base pairs with complementary RNA. These include as non-limiting examples phosphodiesters, phosphorothiodiesters, methylphosphonodiesters and methylphosphonothiodiesters, which provide for increased resistance to degradation after administration. The nucleotides of the antisense oligonucleotide can be 2'-deoxynucleotides or 2'O-methyl nucleotides.

5.1 DETERMINATION OF THE SEQUENCE OF THE ANTISENSE OLIGONUCLEOTIDE

The sequence of RSV strain A is given in Figures 1:1-1:10, in the 5'→3' orientation. The present invention is exemplified by oligonucleotides directed towards strain A2, but the invention can be practiced with any other strain of RSV, having a known genomic sequence. The sequence of the RSV antigenome RNA can be derived therefrom by routine techniques. RSV is a negative strand RNA virus having multiple genes, i.e., the virion contains the complement of the coding strand. On entry into a host cell the genome is transcribed to produce the various mRNA encoding the viral proteins and also to produce an entire complementary RNA, the RSV antigenome, from which the genomic strands of the progeny virus are transcribed. According to the invention the sequence of the antisense oligonucleotide is selected so that the activator-antisense complex binds to and thereby causes the catalytic destruction of the RSV antigenome or alternatively an mRNA. As used herein the terms "antigenomic strand," "RSV antigenome" and "RSV mRNA" are synonyms.

Thus, in an embodiment of the invention the sequence of the antisense oligonucleotide of the invention is selected so that the antisense oligonucleotide is complementary to a portion of the RSV antigenome and will bind to it, i.e., the

activator-antisense complex targets activated RNase L to the portion of the RSV antigenome complementary to the antisense oligonucleotide. Single stranded RNA molecules have regions in which the polymer "folds back" by self hybridizing. These regions of self hybridizing duplex RNA ("stems") are separated by single-stranded "loops" and "bubbles." Thus, not all portions of the RSV antigenome are susceptible to binding to the antisense oligonucleotide with equal affinity and, thus, not all portions of the RSV antigenome are suitable as targets of the activator-antisense complexes.

Which portions of an RNA molecule are in stems and which are in loops or bubbles for the purposes of the invention is determined by a computer modeling program such as "FoldRNA" or "MFOLD", which are in the public domain (e.g., through the Biocomputing Office, Biology Department, Indiana University, Bloomington, IN). Such programs systematically assess all possible conformations and determine the conformation that is the most thermodynamically favored, i.e., has the lowest "free energy." Routinely, conformations that have a free energy within 5% or 10% of the optimal conformation are also determined. Most often these nearly optimal conformations are closely related to each other, for example the position of a small bubble can differ by one or two nucleotides. As used herein a RNA strand is said to be "normally single stranded" when it is single stranded in the conformation having the lowest free energy or a free energy equivalent to the lowest free energy.

The algorithm that is implemented by these programs is described in Zuker et al., 1989, SCIENCE 244:48. The number of steps needed to calculate the lowest free energy state of a polynucleotide, according to the algorithm of Zuker is proportional to the cube of length of the polynucleotide. At present, conformations of 2 KB polynucleotides can be routinely calculated while the calculations of polynucleotides that are the length of the entire RSV antigenome (≈15 KB) are burdensome.

However, because of the kinetics of the intramolecular hybridization of polynucleotides, it is unlikely that conformations involving hybridization between widely separated portions of the polynucleotide do in fact occur even if the modeling programs indicate that they would yield a lower free energy state. Thus, no practical purpose is served by calculating the thermodynamically most stable conformation of the entire RSV antigenome. Rather, for the purposes of the invention, the conformation of the RSV antigenome can be calculated using fragments that are about 1-2 KB in length. If the predicted conformation of a particular portion of the RSV antigenome is dependent upon the length or the boundaries of the nucleotide fragment that is modeled, then the modeling program of the shorter fragment, greater than 1 KB in length, and the fragment wherein the portion is located closest to the middle of the fragment is considered to be the "normally" occurring conformation.

There are several major considerations in selecting which portions of the antisense genome are suitable as targets.

1. Since the RNase L is active only on single-stranded sequences and not on double-stranded sequences, it is important that there be significant stretches of non-base-paired or minimally base-paired nucleotides near the chosen RNA target sequence.

2. Since the RNase L prefers cleavage after UNp sequences, it is preferred that the single-stranded region where cleavage may occur should contain uridine. This is preferred but not essential as it has been shown that the activator-antisense complex can direct cleavage to other nucleotides. Maran et al., 1994.

3. Since cleavage occurs on the 5'-side of the RNA target sequence, it is preferred that such uridine-containing single-stranded regions should be on the 5'-side of the target sequence.

4. Since the antisense domain of the activator-antisense complex must form a double-helical complex with an RNA target sequence, it is preferable that such a targeted sequence be located in a single-stranded or predominantly singly-stranded region of the target RNA. This is due to the consideration that such complex formation is an equilibrium process, and the magnitude of association constant for the process is reduced according to the degree and stability of secondary structure within the specific target sequence.

5. For the reasons expressed in (4) above, Zuker's MFOLD algorithm is used to generate a group of plausible RNA secondary structures. A set of structures can be generated using this program which differ only slightly in energy. Typically the folding program generates secondary structures differing in increments of 0.1 Kcal/mol, and are therefore are energetically very similar.

6. Consideration of (1-5) above leads to a search for the most preferred target sequence in an RNA target. This target ideally should be single-stranded throughout the entire sequence that serves as the antisense binding site as well as a region upstream on the RNA of at least 16 and preferable at least 21 nucleotides. Thus in the ideal situation the preferred target site should be the length of the antisense domain (e.g., 18) plus 16 equals 34 nucleotide in length. Thus, a search would be made for regions in a potential target RNA for single-stranded regions at least 34 nucleotides long and more preferably at least 45 nucleotides long.

7. One additional preference in the design of the activator-antisense complex relates to the composition of the antisense oligonucleotide. Because the activator-antisense complex operates catalytically, there must exist a necessary mechanism for the dissociation of the complex from its complementary sequence in the target RNA. Thus, it is to be expected that duplexes with a large fraction of GC base pairs would undergo dissociation with more difficulty than those

having a large fraction dA-rU or dT-rA pairings. This consideration would also be a preferred design consideration.

Figure 2A shows the results of the modeling of residues 7900-8800 of the mRNA or antigenomic strand. Figure 2A also contains indications of the locations of the antisense oligonucleotides that were tested in the Examples below. Figures 2B:1-2H:3 show alternative results of modeling residues segments of the RSV antisgenome from 1 to 7999, 1100 to 2400 and 2200 to 3300, respectively. Two or three different models of each region, with virtually equivalent energies, are shown. These plots indicate, for example, that preferred embodiments the invention target residues 2490-2530, which is single stranded in all three models, residues 617-663, 3212-3247 and 5240-5288 which are single stranded in at least two of the models shown, and residues 718-772, which is single stranded in one of the three models. It must be remembered that the entire family of generated models differ only by 1.1 kcal/mol, and, therefore, each model represents conformations that can be assumed by the RSV antigenome.

5.2 THE STRUCTURE OF THE ACTIVATOR

Examples of the structure of the activator are described in patent publication WO94/09129, at pages 10, 45 and 46-51, which is hereby incorporated by reference. Briefly, the activator can contain at least three riboadenylate residues, linked by 2'-5'phosphodiester bonds, having a free 5' mono-, di- or triphosphate or thiophosphate. The 5' thiophosphate-tetra-adenylate activator (sp5'A2'(p5'A2')$_3$-O-) is the preferred activator. Other activators include p5'A2'(p5'A2')$_2$-O-, sp5'A2'(p5'A2')$_2$-O-, and p5'A2'(p5'A2')$_3$-O-.

Phosphorothioate and phosphorodithioate linkages between adenine nucleosides can be used as well as phosphodiester. The use of these linkages results in decreased degradation but also decreased activity. Beigelmann, L., et al., 1995, Nucleic Acid Research 23:3989-94. The use of a 5'-thiophosphate results in greatly improved activity and

stability. Those skilled in the art appreciate that other nucleotides can be attached to the 3'hydroxyl or 2'hydroxyl of the 2'-5'tri- or tetra-adenylate without changing its activity as an RNase L activator. Thus, these embodiments are also included in the scope of the term "activator of RNase L." Those skilled in the art will further recognize that oligonucleotides containing bases other than adenine, such as inosine at the second nucleotide (counting 5'→3') can also be used. Those skilled in the art also recognize that non-nucleotide activators of RNase L can be used in the invention and are equivalents of nucleotide activators. As used herein the term "2-5A" refers to any nucleotide activator of RNase L and the term "activator of RNase L" refers to any activator of RNase L including 2-5A. The term 2',5'A refers specifically to 2',5'-linked oligoadenylates.

5.3 THE STRUCTURE OF THE ANTISENSE OLIGONUCLEOTIDES

The antisense oligonucleotide can have any structure now known or to be developed in the antisense art. These include phosphodiesters, phosphorothiodiesters, methylphosphonodiesters and methylphosphonothiodiesters, which provide for increased resistance to degradation after administration. The nucleotides of the antisense oligonucleotide can be 2'-deoxynucleotides or 2'O-methyl nucleotides.

The preparation of modified and unmodified oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) Trends Biotechnol. 10:152-158; Agrawal in Protocols for Oligonucleotides and Analogs, Synthesis and Properties (Agrawal, ed.), Humana Press, Totowa, New Jersey (1993), Chapter 20). For example, nucleotides can be covalently linked using art-recognized techniques such as phosphoramidate, H-phosphonate chemistry, or methylphosphoramidate chemistry (see, e.g., Uhlmann et al. (1990) Chem. Rev. 90:543-584; Agrawal et al. (1987) Tetrahedron. Lett. 28:(31):3539-3542); Caruthers et al. (1987) Meth. Enzymol. 154:287-313; U.S. Patent 5,149,798).

Oligomeric phosphorothioate analogs can be prepared using methods well known in the field such as methoxyphosphoramidite (see, e.g., Agrawal et al. (1988) Proc. Natl. Acad. Sci. (USA) 85:7079-7083) or H-phosphonate (see, e.g., Froehler (1986) Tetrahedron Lett. 27:5575-5578) chemistry. The synthetic methods described in Bergot et al. (J. Chromatog. (1992) 559:35-42) can also be used.

5.4 THE STRUCTURE OF THE LINKER

Any linker that covalently connects an activator of RNase L and the antisense oligonucleotide and does not prevent the activator from activating RNase L can used. In a preferred embodiment the linker is attached to the 3' or 2' terminus of a 2-5A activator. In a further preferred embodiment the linker consists of a bis-1,4-butanediol-phosphodiester which connects the 3' or 2' terminus of a 2-5A activator and the 5' or the 3' terminus of the antisense oligonucleotide. Attachment to a terminus of the antisense oligonucleotide is selected for the convenience of synthesis. Those skilled in the art appreciate that attachment to an internal 2' hydroxyl or to a portion of the nucleotide base that is not critical to base pairing are alternative embodiments of the invention.

5.5 USE OF THE ACTIVATOR-ANTISENSE COMPLEXES

The activator-antisense complexes of the invention can be administered to a subject having an RSV infection by any route effective to deliver the activator-antisense complexes to the epithelium of the bronchi, bronchioles and alveoli of the subject. In one embodiment the activator-antisense complexes are delivered by use of an inhaled aerosol, according to the techniques well known in the art for the delivery of ribavirin. In a further embodiment of the invention a mixture of ribavirin and an activator-antisense complex of the invention can be administered in a common pharmaceutical carrier.

In an alternative embodiment the activator-antisense complex can be administered parenterally, e.g., by intravenous infusion. When delivered by intravenous administration, the dose of activator-antisense complex can be determined by routine methods well known to pharmacologists so that the serum concentration approximates the concentration at which antiviral activity is seen in the *in vitro* examples described below, e.g., a concentration of about 10 μM of spA_4-antiRSV3'-3'T/(8281-8299). When delivered by aerosol administration the dose should be selected so that the tissue concentration in the lung approximates the concentration at which antiviral activity is seen in the *in vitro* examples.

EXAMPLES

6. MATERIALS AND METHODS

Sequence conventions. The practice of the RSV literature, position 1 of the RSV genome (the virion RNA) is the 3' terminus; position 1 of the RSV antigenome (mRNA) is the 5' terminus. Thus, for example, the antisense oligonucleotide labeled antiRSV/(8490-8509) has the sequence (5'→3') of residues 8509 to 8490 of the RSV genome and is complementary to residues 8490-8509 of the RSV antigenome. Note, however, that the RSV strain A2 genome sequence of Figures 1:1-1:10 is in conventional 5' to 3' order. Hereinafter activator-antisense complexes wherein the activator is a 2',5'A are termed "2-5A antisense chimeras."

Synthesis and purification of 2-5A antisense chimeras.

Oligonucleotide Structural Types Synthesized. The following generic oligonucleotide types were prepared for this study.

I. p5'A2'p(5'A2'p)$_3$-[O(CH$_2$)$_4$Op]2-5'dN3'p(5'dN3'p)$_n$5'dN

II. A2'p(5'A2'p)$_3$-[O(CH2)$_4$Op]$_2$-5'dN3'p(5'dN3'p)$_n$5'dN

III. dN3'p(5'dN3'p)$_n$5'dN

IV. p5'A2'p(5'A2'p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5'dN3'p(5'dN3'p)$_m$5'dN3'p-3'pdN5'

V. sp5'A2'p(5'A2'p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5'dN3'p(5'dN3'p)$_m$5'dN3'p-3'pdN5'

VI. A2'p(5'A2'p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5'dN3'p(5'dN3'p)$_m$5'dN3'p-3'pdN5'

VII. sp5'A2'p(5'A2'p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5'dN3'p(5'dN3'p)$_n$5'dN

VIII. p5'A2'p(5'A2'p)$_3$-[O(CH$_2$)$_4$Op]$_2$-3'dN5'(p3'dN5')$_n$p3'dN

The following procedures are illustrative of those employed to synthesize the 2-5A-antisense chimeric oligonucleotides in classes I - VIII above. In general, they follow the synthetic strategy developed in Lesiak et al., 1993.

Reagents and Chemicals Employed.

1. For initiation of synthesis on solid support:

dA-3'-lcaa-CPG (500 Å)
5'-O-dimethoxytrityl-N6-benzoyl-2'-deoxyadenosine-3'-lcaa-CPG

dC-3' lcaa-CPG (500 Å)
5'-O-dimethoxytrityl-N4-benzoyl-2'-deoxycytidine-3'-lcaa-CPG

dG-3' lcaa-CPG (500 Å)
5'-O-dimethoxytrityl-N2-isobutyryl-2'-deoxyguanosine-3'-lcaa-CPG

dT-3'-lcaa-CPG (500 Å)
5'-O-dimethoxytritylthymidine-3'-lcaa-CPG

These solid supports were used to synthesize oligonucleotides with the normal 3'→5' phosphodiester bonds. All were 1 μmole size. These DMT protected nucleosides are attached to controlled pore glass (CPG) through a succinyl group and a long chain alkyl amine (lcaa) linker are commercially available products of Applied Biosystems (Foster City, CA). These supports were employed in the synthesis of generic oligonucleotide types I, II, III, and VII.

dA-5'-lcaa-CPG (500 Å)
3'-O-dimethoxytrityl-N6-benzoyl-2'-deoxyadenosine-5'-lcaa-CPG

dC-5' lcaa-CPG (500 Å)
3'-O-dimethoxytrityl-N4 -benzoyl-2'-deoxycytidine-5'-lcaa-CPG

dG-5' lcaa-CPG (500 Å)
3'-O-dimethoxytrityl-N2-isobutyryl-2'-deoxyguanosine-5'-lcaa-CPG

dT-5'-lcaa-CPG (500 Å)
3'-O-dimethoxytritylthymidine-5'-lcaa-CPG

These solid supports were obtained form Glen Research (Sterling, VA) and were used to synthesize oligonucleotides with the reversed polarity 5'→3' phosphodiester bonds. All were 1 μmole size. These supports were employed for the synthesis of generic oligonucleotide types IV, V, VI, and VIII.

2. Elongation of the DNA antisense chain.

For normal 3'→5' phosphodiester bond oligonucleotides, a total of 500 mg of each of the following phosphoramidites (Applied Biosystems) was dissolved in the indicated amount of anhydrous acetonitrile to make a 0.1 M phosphoramidite solution:

5'-O-dimethoxytrityl-N6-benzoyl-2'-deoxyadenosine-3'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (5.6 mL)

5'-O-dimethoxytrityl-N4-benzoyl-2'-deoxycytidine-3'(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (5.9 mL)

5'-O-dimethoxytrityl-N2-isobutyryl-2'-deoxyguanosine-3'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (5.8 mL)

5'-O-dimethoxytrityl-2'-deoxythymidine-3'-(2-cyanoethyl-N,N-diiso propyl)phosphoramidite (6.6 mL)

The foregoing were used in the preparation of generic oligonucleotide types I, II, III, IV, V, VI, and VII.

For the synthesis of oligonucleotides with all DNA phosphodiester bonds with reversed polarity, the following phosphoramidites were obtained from Glen Research (Sterling, VA).

3'-O-dimethoxytrityl-N6-benzoyl-2'-deoxyadenosine-5'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (5.6 mL)

3'-O-dimethoxytrityl-N4-benzoyl-2'-deoxycytidine-5'(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (5.9 mL)

3'-O-dimethoxytrityl-N2-isobutyryl-2'-deoxyguanosine-5'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (5.8 mL)

3'-O-dimethoxytrityl-2'-deoxythymidine-5'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (6.6 mL)

The above intermediates were employed to synthesize generic oligonucleotide type VIII.

3. Linker to join chimeric domains.

The linker, (2-cyanoethyl-N,N-diisopropyl)-[4-O-(4,4'-dimethoxytrityl) butyl]phosphoramidite, was synthesized by a modification of an earlier described procedure (Lesiak et al., 1993), and a 0.1 M solution was made by dissolving 100 mg linker in 1.7 mL of anhydrous acetonitrile.

4. For synthesis of 2',5'-oligoadenylate domain of the chimera.

5'-O-dimethoxytrityl-N6-benzoyl-3'-O-t-butyldimethylsilyladenosine-2'-N,N-di-isopropylcyanoethylphosphoramidite (ChemGenes Corp., Waltham, MA, cat no. ANP 5681). A 0.1 M solution was made by dissolving 500 mg of monomer in 5.0 mL of anhydrous acetonitrile.

5. Phosphorylation Reagent for 5'-terminus of 2',5'-oligoadenylate domain of chimera.

2-[2-(4,4'-dimethoxytrityl)ethylsulfonyl]ethyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (Glen Research, Sterling, VA. cat no. 10-1900-90) was used at a concentration of 0.2 M in anhydrous tetrazole/acetonitrile (ABI) for semi-automated synthesis.

6. Other Reagents.

All other DNA synthesis reagents were obtained from Applied Biosystems Inc. which includes diluent (acetonitrile), activator solution (tetrazole/acetonitrile),

capping solutions (A: acetic anhydride solution and B: N-methylimidazole solution), deblocking reagent (trichloroacetic acid solution), oxidizer (iodine solution), and tetraethylthiuram disulfide sulfurization reagent.

Tetrabutylammonium fluoride in tetrahyrofuran (Aldrich, Milwaukee, WI) was used to deblock the t - butyldimethylsilyl group used for protection of the 3'-hydroxyls of (2',5')-oligoriboadenylate domain.

7. SYNTHESIS PROCEDURE

The 2',5'-oligoadenylate/antisense chimeras were synthesized by modified automated or semi-automated procedure.

All of the chemicals were dried over P_2O_5 in vauco overnight before use. The 1 μmole deoxynucleoside-lcaa-CPG column was used.

The core (2',5')-oligoadenylate/antisense chimera refers to the complete 2',5'A-antisense chimera minus the 5'-terminal monophosphate group and has three regions defined for synthetic purposes: an antisense region, a linker region, and (2',5')-oligoadenylate region. The 2',5'A-antisense chimera was synthesized by the automated method listed in Table 1.

1 μmole scale standard synthesis cycle was used. The cycle was modified by changing the coupling time (coupling of monomer) for each different region. The monomer/ acetonitrile solution was installed on the DNA synthesizer by a double change procedure to avoid contaminants. After the synthesis of each region, the column was dried completely by Argon for at least 3 min. and the synthesis cycle, trityl mode, and sequence were edited for the synthesis of next region of the desired oligonucleotide.

For preparation of core 2',5'A-antisense chimeras without a 5'-monophosphate group, the final step was omitted in Table 1. For semi-automated preparation of the 5'-monophosphate terminating chimeras, the core oligonucleotide was synthesized with the trityl group on, and the column was

dried and removed from the DNA synthesizer. The 5'-end phosphorylation was performed manually according to the procedure presented in Table 2.

Cleavage and Deprotection

1. The oligonucleotide was cleaved from the CPG support by concentrated ammonium hydroxide/ethanol (3:1 v/v) at room temperature for 2 hours.

2. The ammonium hydroxide/ethanol solution of crude oligonucleotide was removed into a 3 mL vial and sealed tightly. The solution was incubated at 55°C for 8 hours to remove the protecting groups on the bases.

3. The resulting ammonium hydroxide/ethanol solution of oligonucleotide was transferred to a glass tube, and cooled completely in a ice-bath. The solution was then evaporated to dryness in a speedvac concentrator and a solution of tetrabutylammonium fluoride (2 mL, 1.0 M) in THF was added, and the entire mixture was vortexed for at least 1 min. This reaction mixture was allowed to incubate at room temperature for at least 10 hours.

An equivalent volume of 0.1 M TEAA (tetraethylammonium acetate) (pH 7.0) buffer was added, mixed and evaporated to half volume to remove THF. The residue was subjected to purification by HPLC.

Purification of the Oligonucleotides

1. Polystyrene Reverse-Phase Ion-Pair Chromatography (PRP-IPC) Protocol (a modification of the method of Swiderski, et al., 1994).

The oligonucleotide was dissolved in about 4 - 5 mL water to make a clear solution (centrifuged if necessary), and the clear solution was directly injected into the PRP-1 HPLC column (300 x 7 mm). The reaction mixture was thus simultaneously desalted and purified.

Solvent A: 10 mM tetrabutyl ammonium phosphate (TBAP), pH 7.5 in water.

Solvent B: 10 mM TBAP, pH 7.5 in acetonitrile/water (8:2 v/v).

The sample was eluted with a convex gradient of 5 - 90% solvent B in A in 60 min. at a flow rate of 1.5 mL/min.

Fractions containing desired oligo were pooled and evaporated to about 1 - 2 mL. The oligo-TBA ion-pair was converted into its sodium salt form by the following procedure:

1 mL of Dowex 50W ion exchange wet resin (Na^+ form) was added into oligonucleotide/water solution. The solution was stirred for at least 30 min. in the cold room. The resin was removed by passing the solution through a Poly-Prep chromatography column (Bio-Rad, Cat. # 731-1550). The resin was washed with extra water until no oligonucleotide remained on the resin.

Alternately, prior to Dowex treatment the oligonucleotide was passed through a C-18 Sep-Pak cartridge according to the following procedure.

a. The C-18 cartridge was pre-washed with 10 mL methanol and 10 mL water.
b. The oligo solution was loaded onto the cartridge.
c. The cartridge was washed with 20 mL water to remove salt from the column.
d. The oligonucleotide was eluted with 10 mL of 50% methanol in water.
e. The desalted oligonucleotide was detected by UV spectrophotometer and the fractions containing oligo were combined and concentrated.

Dialysis of (2',5')-Oligoadenylate/antisense Chimeras

After Purification by HPLC and ion exchange, the oligonucleotide (sodium salt) was dialyzed to remove small molecules and excess salt. The dialysis was carried out at 4°C. The oligonucleotide was dialyzed against 0.02 M NaCl

first for 4 - 6 hours and then against water for 48 hours. If the oligonucleotide was desalted on C-18 sep-pak cartridges after HPLC purification, the time of dialysis can be shortened to 6-10 hours.

Post-treatment of Oligoadenylate/antisense Chimeras

The oligonucleotide, after dialysis, was passed through a 0.22 μ millex-GV filter unit (Millipore, Cat. No. SLGV025LS) for sterilization. The resulting solution was quantitated as O.D. A260 by UV/Vis spectrophotometry.

Nucleotide composition analysis of (2',5')-Oligoadenylate/antisense Chimeras

1. Nucleotide Composition Analysis

The nucleotide composition of the chimeric oligonucleotide were analyzed by enzymatic digestion with snake venom phosphodiesterase (*Crotallus durissus*) (Pharmacia, cat # 27,0821-01).

A purified oligonucleotide (0.2 A260 O.D.U.) is incubated with snake venom phosphodiesterase (0.15 units) in 50 mM Tris/HCl, pH 8.0, 0.5 mM $MgCl_2$, pH 8.0. The 100 μL mixture was incubated at 37°C for at least 3 hours. For chimeric oligonucleotides containing a 3'-3'dN, such as Oligonucleotide Structural Type IV (section 6), the incubation time was extended to 10 hours.

After digestion, the solution was treated with Microcon-10 (Amicon, Inc. product No. 42406). The microcon was first spin-rinsed with water before addition of 100 μL sample solution. The centrifuge time was typically 45 min. The clear solution was used for HPLC analysis.

An aliquot (5 - 10 μL) of the hydrolysate was analyzed by reverse phase HPLC using a Beckman Ultrasphere C-18 ODS column (0.46 x 25 cm). Separation of the digestion products was accomplished under the following conditions: 2% B isocratically for 20 min. linear gradient 2 - 50% B for 15 min. and held isocratically 10 min where solvent A was 100 mM ammonium phosphate, pH 5.5 and solvent B was methanol/water

(1:1 v/v). The flow rate was 0.5 mL/min. The standard markers dCMP, TMP, dGMP, AMP and dAMP (Aldrich Chem. Co.) were used to compare retention times and elution orders of the hydrolysis products. Typically, the peaks obtained from the enzymatic hydrolysis of an oligonucleotide had retention times of 9.7 min. (dCMP), 27.3 min. (TMP), 29.6 min. (dGMP), 31.7 min. (AMP), 39.5 min. (Alinker) and 41.2 min. (dAMP). The retention times varied depending on the column, pH value of mobile phase and the equilibrium times of the column. The integrated peak areas provided the relative content of each nucleotide. The extinction coefficients of 7610 (dCMP), 8158 (TMP), 9969 (dGMP), 12342 (AMP & Alinker), 14361 (dAMP) measured at 260 nm in 100 mM ammonium phosphate, pH 5.5 were used in the analysis.

Oligonucleotide Purity Confirmation

The purities of (2',5')-oligoadenylate/antisense chimeras were checked by HPLC or gel capillary electrophoresis (GCE). The purity was obtained by the integration of peak area detected at 260 nm.

1. Gel Capillary Electrophoresis (GCE) Method

The measurement of oligonucleotide purity was performed on an Applied Biosystems 270A-HT capillary electrophoresis instrument using MICRO-GEL100 (Applied Biosystems Inc.) gel filled capillaries (50 uM i.d., effective length 27 cm, running buffer, 75 mM Tris phosphate (pH 7.6), 10% methanol). Detection was at 260 nm. A typical electrophoregram of (2',5')-oligoadenylate/antisense chimera was obtained by the following conditions: sample concentration was approx. 0.1 O.D./mL, electrokinetic injection was 2 s at -5kv. Voltage was -14 mA (19 mA) and the operation temperature was 30°C. Under this condition, the (2',5')-oligoadenylate/antisense chimera had about 1 min. earlier elution time than that of its core analogue.

2. Dionex PA-100 Ion Exchange HPLC Method

The purities of oligonucleotides could also be measured by a Dionex Ion exchange HPLC. Usually, the dionex PA-100 ion exchange column could provided higher resolution and better peak shape compared with other HPLC chromatographic method for the analysis of (2',5')-oligoadenylate/antisense chimera.

A typical chromatogram of (2',5')-oligoadenylate/antisense was obtained by the following conditions: Dionex PA-100 (4 x 250 mm) column (Dionex, cat # 43010). Solvent A was 25 mM Tris/HCl and 0.5% acetonitrile (pH 7.0), solvent B was 25 mM Tris/HCl, 0.5% acetonitrile and 1 M ammonium chloride (pH 7.0). The sample was eluted in linear gradient of 10 - 70% B in A during 30 min. and held isocratically for 10 min. at a flow rate of 1 mL/min. Detection was at 260 nm.

Cell culture, RSV propagation and infection, and viral titer assays.

The human tracheal epithelial cell line, 9HTE, (Gruenert et al., 1988) and CV-1 cells, (American Type Culture Collection, Rockville, MD, CCI#70) a green monkey kidney cell line which is highly permissive to RSV infection, were cultured in minimal essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 1X MEM amino acids solution, 1X MEM non-essential amino acids solution, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B ("culture medium") (all reagents from Gibco BRL, Bethesda, MD). RSV strain A_2 (ATCC No. VR1302) was propagated in CV-1 cells. CV-1 monolayers were infected at a multiplicity of infection (M.O.I.) of 0.2 and cultured 46 h in MEM, 2% FBS, 1X Penicillin/Streptomycin (PS) in 5% CO_2, 95% O_2 at 37°C. Cells were then washed 2 times in MEM and subsequently covered in MEM, 2% FBS, 1X PS, 50 mM HEPES (pH7.5), 100 mM $Mg(SO_4)$. After 2 h at 37°C, cells were scraped and sonicated as previously described (Panuska et al., 1995). Aliquots (1 ml each) of cell sonicates were

flash frozen in ethanol/dry ice within 20 min of scraping. Several aliquots were then thawed and titered by a plaque assay on CV-1 cells as previously described (Cirino et al., 1993). The range of virus yield from this procedure was 2 to 7 X 10^6 plaque forming units (pfu) per ml.

Oligonucleotide, interferon α and ribavirin treatments of 9HTE cells before and after RSV infection.

Infection of 9HTE cells was performed as previously described (Merolla et al., 1994). Briefly, confluent monolayers were exposed to RSV diluted in MEM, 2% FBS, for 2 h at 37°C in 5% CO_2, 95% O_2. After exposure, cells were washed two times with serum-free MEM media and then fresh culture media (with 10% FBS) was added. Oligonucleotides were either added 4 h prior to infection (t_{-4}) or immediately after infection (t_{+2}) and also at t_{+14} and t_{+26}. Cells were harvested for plaque assays at 36 h post-infection to determine viral titers as previously described (Cirino et al., 1993). Cells were washed twice to remove any residual antisense and were then scraped in MEM containing 2% FBS, 1X PS. 9HTE cells were sonicated for 20 sec on ice then the extracts were serially diluted and transferred to a confluent monolayer of CV-1 for quantitation of infectious viral particles. CV-1 were exposed to sonicated 9HTE for 2 h then washed once in MEM and overlaid with Eagle's minimal essential medium (EMEM, BioWhittaker, Walkersville, MD) containing 2% FBS, 200 U/ml penicillin, 200 μg/ml streptomycin, 0.5 μg/ml amphotericin B, and 0.4% agarose. Five days later, cells were fixed in 10% formalin for 1 h, the agarose plugs removed, and 0.2% crystal violet in 10% formalin was added for 2 min. CV-1 were subsequently washed in water to remove excess dye and the number of syncytia (plaques) were quantified under a microscope.

In certain experiments (data not shown), interferon α (Schering, Intron A, interferon α-2B, 10^5 U/ml) or Ribavirin (ICN Pharmaceuticals, Costa Mesa, CA, 100 μg/ml) were also added after infection. Interferon α was added to a final

concentration of 50 U/ml when chimeric antisense was added, i.e. t_{+2}, t_{+14} and t_{+26}. In contrast, ribavirin, which has a half-life of 40 days in vivo, was added only at t_{+2} to a final concentration of 10^{-13} M.

Reverse transcriptase-coupled polymerase chain reaction (RT-PCR)

RNA was collected from $2x10^5$ 9HTE cells at 8 h post-infection (M.O.I.=10) by RNAzol treatment as described by the manufacturer (Tel-Test, Inc, Freindswood, TX). RNA was isolated after 8 h to limit RSV replication to a single cycle. Isolated RNA (≈1 μg) was incubated with 100 pmoles of the appropriate downstream (-) primer listed below or 100 pmoles of random hexamer (used for glyceraldehyde-3-phosphate dehydrogenase, GAPDH, mRNA only). RNA and primers were heated to 70°C for 10 min then cooled rapidly on ice for 5 min. A final reaction volume of 30 ml contained: 300 μM each dNTP, 200 U SuperScript reverse transcriptase (GibcoBRL, Bethesda, MD), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 10 mM DTT. Reverse transcription was allowed to proceed for 1 h at 37°C.

PCR reactions were performed using 50 μl Hot-Start tubes (Molecular Bio-Products, San Diego, CA) with 25 μl lower buffer containing 40 mM Tris-HCl (pH=8.4), 100 mM KCl, 2 mM MgCl2, 600 μM each dNTP, and 100 pmoles each of the appropriate primer pairs;

TARGET	SEQUENCE	ANNEALING TEMP
RSV(L+) (Seq ID NO:1)	[5'-TCAATGGTCCTTATCTCAA-3']	46°C
RSV(L-) (Seq ID NO:2)	[5'-GAGCTTTATTAGCAGCATC-3']	
GAPDH(+) (Seq ID NO:3)	[5'-AAATCCCATCACCATCTTC-3']	57°C
GAPDH(-) (Seq ID NO:4)	[5'-CACCACCCTGTTGCTGTAG-3']	
RSV(M2+) (Seq ID NO:5)	[5'-AAACAATCAGCATGTGTTG-3']	46°C
RSV(M2-) (Seq ID NO:6)	[5'-AATGTAACGATGTGGTGAG-3']	

25 μl Hot-Start upper buffer contained 5 U Taq DNA polymerase (GibcoBRL) and 1/10th of the cDNA from the RT reaction. 30 cycles of PCR were performed with 1 min at 92°C, 1.5 min at the annealing temperature indicated above, and 2 min at 72°C. Aliquots of the RT/PCR mixtures were analyzed on 1% agarose/TBE gels.

EXAMPLE 8. RESULTS

2-5A antisense inhibits RSV replication in previously infected human tracheal epithelial cells.

To develop 2-5A antisense chimeras with the potential to block RSV replication, we first selected an oligonucleotide binding site in the viral RNA polymerase (RSV L) mRNA, which encodes a low abundance message that is absolutely required for RSV replication. The first chimera synthesized and evaluated was pA_4-antiRSV/(8490-8509). The binding sites for the chimeric oligonucleotide's antisense domain are to the transcripts of nucleotides 8490-8509 in the RSV genome, which spans the translation start codon for the L protein, and to nucleotides 8490-8509 of the antigenomic strand (the template for reproduction of the genome). Since to function as an effective treatment, a candidate agent must be able to inhibit viral replication subsequent to diagnosis, the anti-RSV effect of the 2-5A antisense chimera was determined on human tracheal epithelial cells, 9HTE, with treatments beginning either 4 h before RSV infection (pre-/post-infection treatments) or 2 h following infection (post-infection treatments). In the post-infection treatments, pA_4-antiRSV/(8490-8509) at (10 μM final concentration), was added at t_{+2}, t_{+14} and t_{+26}, (numbers represent time in h relative to time of infection, t_0). For pre-infection treatment, pA_4-antiRSV/(8490-8509) (10 μM final concentration), was added at t_{-4}, and t_0 in addition to t_{+2}, t_{+14} and t_{+26}. Virus harvested from control and oligonucleotide-treated 9HTE cells was measured by infecting CV-1 cells and subsequently counting viral plaques (see Materials and Methods). Post-infection treatment of 9HTE cells with pA_4-antiRSV/(8490-8509) was found

to be just as effective as pre-/post-infection treatment; both resulted in about 70% inhibition of RSV replication. On the basis of these experiments, all subsequent experiments were performed with post-infection treatments only. Additionally, these experiments indicate the potential use of these compounds as a treatment for active infection as compared to a prophylactic measure.

Antiviral activities of 2-5A antisense and control chimeric oligonucleotides directed against the viral L polymerase mRNA translation start site.

An initial series of oligonucleotides included various controls and additional modifications designed to stabilize the chimeras against enzymatic decay in the cell culture (Table 3). To compare the antiviral effects of these oligonucleotides, 9HTE cells were infected with RSV and subsequently treated three times (t_{+2}, t_{+14}, and t_{+26} h) with three concentrations of oligonucleotides (3.3, 6.6 and 9.9 μM) and virus was harvested after 36 h. Chimeric antisense lacking only the 5'-phosphate, in A_4-antiRSV3'-3'C/(8490-8509), deficient in the ability to activate RNase L (Maran et al., 1994), was used as a control. This derivative showed only minimal anti-RSV activity (28.3% inhibition at 9.9 μM/treatment as compared to 64.8% inhibition by the 5'-phosphorylated derivative, pA_4-antiRSV3'-3'C/(8490-8509)). To stabilize the 3' termini of the chimeras, these ends were masked. In one derivative, pA_4-3'antiRSV5'/(8490-8509), the 2-5A portion of the chimera was linked to the 3' end of the antisense moiety instead of to the 5' end of the oligonucleotide. In this way, the 3' terminus of the antisense is protected from exonuclease digestion though its attachment to the linker (G.L., W.X., & P.F.T., unpublished observations). This analog produced a 69% inhibition of virus production at the highest concentration tested (9.9 μM) (Table 3). In another chimera, pA_4-antiRSV3'-3'C/(8490-8509), the 3' terminal deoxynucleotide was connected by a 3'-3' phosphodiester linkage to the penultimate deoxynucleotide

thereby slowing 3' exonuclease digestion (G.L., W.X., & P.F.T., unpublished observations). This compound produced a 1.6-fold enhanced antiviral activity at 6.6 μM (64.3% inhibition) compared with the standard, unmodified chimera, pA_4-antiRSV3'-3'C/(8490-8509) tested at the same concentration (38.8% inhibition). Alternately, a 5'-thiophosphate was used to stabilized the 2-5A domain of the chimera against phosphatase. Such thiophosphorylated derivatives of 2-5A and 2-5A antisense were previously shown to be fully capable of activating RNase L when compared to standard, 5'-phosphorylated, 2-5A and 2-5A antisense (Xiao et al., 1994 and Maran et al., 1994). spA_4-antiRSV/(8490-8509) showed a substantially increased anti-RSV effect with 71% and 94% inhibition of viral growth at treatment concentrations of 6.6 and 9.9 μM, respectively (Table 3).

EXAMPLE 9. SELECTION OF TARGET

Selection of highly effective 2-5A antisense chimera based on a computer analysis of the RNA secondary structure.

A computer-assisted analysis of the secondary structure of the RSV mRNA was performed to identify single-stranded regions as oligonucleotide binding sites. Computer prediction of the secondary structure of RSV antigenomic strand, nucleotides 7900 to 9079, including a 3' portion of the M2 gene, encoding a viral envelope protein, and a 5' region of the L gene, was performed using the program MFOLD which finds a secondary structure of minimum free energy for an RNA molecule based on published values of stacking and loop destabilizing energies. MFOLD is the program of Michael Zuker (Zuker, 1989). The energies used by Zuker's program were first described by Salser (1977) and are now defined by Turner and colleagues (Freier et al., 1986). The analysis showed a large loop from positions 8250 to 8299. This loop was present in a 90 codon open reading frame of unknown function downstream (3') of the major M2 open reading frame. Three chimeric compounds were synthesized which were

complementary to sequences in the loop, spA_4-antiRSV3'-'3A/(8251-8270), spA_4-antiRSV3'-3'T/(8261-8279), and spA_4-antiRSV3'-3'T/(8281-8299). In addition, three oligonucleotides were synthesized to other regions in RNA that included a bulge, a hair-pin and a small loop, spA_4-antiRSV3'-3'A/(8530-8547), spA_4-antiRSV3'-3'C/(8561-8578), and spA_4-antiRSV3'-3'G/(8599-8618), respectively. When added to the infected 9HTE cells at concentrations of 3.3 μM, the three oligonucleotide directed to the large loop had the greatest level of antiviral activity (78 to 91% inhibition) (Table 4). These three oligonucleotides had very substantially improved anti-RSV activity compared to the previously described 2-5A antisense molecules (3 to 16.5% inhibition at 3.3 μM, see Table 3). The chimera with the greatest anti-RSV effect was spA_4-antiRSV3'-3'T/(8281-8299), which produced 97 and 99.6% inhibition of RSV replication at doses of 6.6 and 9.9 μM, respectively. The oligonucleotide directed to the region in the RNA with the bulge, spA_4-antiRSV3'-3'A/(8530-8547), showed almost no antiviral effect at 3.3 μM (Table 4). The 2-5A antisense molecules to the hairpin and small loop, spA_4-antiRSV3'-3'C/(8561-8578), and spA_4-antiRSV3'-3'G/(8599-8618), had intermediate activities, 57 and 43% inhibition of RSV replication at concentrations of 3.3 μM.

Figure 3 presents a comparison of spA_4-antiRSV3'-3'T/(8281-8299) and spA_2-antiRSV3'-3'T/(8281-8299). Only the tetraadenylate is an activator of RNase L, hence the greater potency of the spA_4-linked oligonucleotide compared to the spA_2-linked oligonucleotide establishes the role of RNase L activity in the protective effects of the present invention.

EXAMPLE 10. BIOCHEMICAL ANALYSIS OF RESULTS

Correlation of antiviral activities and RNA levels after treatment of RSV-infected 9HTE cells with the 2-5A antisense chimeras.

To determine if RSV RNA levels correlated with antiviral activity, RT-PCR analysis was performed on RNA isolated from RSV-infected and uninfected 9HTE cells with and without treatment with spA_4-antiRSV3'3'T/(8281-8299) or spA_4-antiRSV3'3'A/(8530-8547). An M2 RNA sequence in RSV (from nucleotides 7879 to 8465) was converted to cDNA and amplified by PCR (Materials and Methods). M2 RNA from the RSV-infected cells produced an RT-PCR DNA product that was clearly visible. In contrast, there was no M2 RNA detected from the RSV-infected cells treated with spA_4-antiRSV3'3'T/(8281-8299). The chimera directed against the RSV L mRNA and the corresponding sequence in the antigenomic RNA, spA_4-antiRSV3'3'A/(8530-8547), had little effect on levels of M2 RNA (17% inhibition). Accordingly, the levels of viral M2 RNA were dramatically reduced in 9HTE cells treated with spA_4-antiRSV3'-3'T/(8281-8299) while those treated with a relatively inactive control chimera against the RSV L mRNA, spA_4-antiRSV3'-3'A/(8530-8547), had no affect on levels of M2 RNA. Levels of GAPDH transcripts were similar in all of these RNA preparations. These results showing loss of the specific RSV mRNA target are consistent with involvement of RNase L.

EXAMPLE 11: OTHER ACTIVATOR-ANTISENSE COMPLEXES

The secondary structure of the 5' terminus of the RSV antigenomic strand can be more readily disrupted than the internal portions. Thus, the following activator-antisense complexes can be used to practice the invention despite the absence of large loops in modeling of the secondary structure of the antigenomic strand.

spA_4-antiRSV3'-3'T/(1-19): (Seq ID NO:7)	sp5'A2'(p5'A2')$_3$-[(Bu)p]$_2$-(5'ttg tac gca ttt ttt cgc g3'-3't5')
spA_4-antiRSV3'-3'T/(51-69): (Seq ID NO:8)	sp5'A2'(p5'A2')$_3$-[(Bu)p]$_2$-(5'gta ctt atc aaa ttc tta t3'-3't5')

At the 3'-terminus of the RSV genome, there is a block of about 50 nucleotides which is not incorporated into the protein encoding transcript of the 3'-proximal gene but which is transcribed to yield a small RNA species that has been called a "leader RNA." Evidence indicates that the exact 3'-end of the genome is the entry site for the RNA transcriptional machinery and that leader RNA synthesis, which involves termination at a purine-rich sequence at the leader-template-NP-gene boundary, is an obligatory prelude to progression of the transcriptase through the rest of the genome. In addition, since the 3'-end of the genome is where both replicative and transcriptional RNA synthesis initiate, this site provides a site at which the critical switch between the two kinds of RNA synthesis may operate. Finally, the 3'-terminus of the RSV genome is rich in uridylate residues which may be more readily susceptible to cleavage by the 2-5A-dependent RNase.

These functions of the 3'-terminus of the genomic strand can be disrupted more readily than other portions of the genomic strand. Thus the following activator-antisense complexes, which bind to the genomic strand can be used to practice the invention.

spA_4-antiRSVGe3'-3'T/(1-18): sp5'A2'$(p5'A2')_3$-$[(Bu)p]_2$-(5'acg cga aaa aat gcg tac3'-3't5') (Seq ID NO:9)

spA_4-antiRSVGe3'-3'T/(84-101) (Seq ID NO:10):
sp5'A2'$(p5'A2')_3$-$[(Bu)p]_2$-(5'ctc cct tgg tta gag atg3'-3't5')

spA_4-antiRSVGe3'-3'T/(369-386) (Seq ID NO:11):
sp5'A2'$(p5'A2')_3$-$[(Bu)p]_2$-(5'gaa atg atg gaa tta aca3'-3't5')

EXAMPLE 12: COMPARATIVE DATA OF spA_4-antiRSV3'3'T/(8281-8299)

A comparison of the efficacies of spA_4-antiRSV3'3'T/(8281-8299) treatement and conventional

ribarvirin treatement can be obtained by determining the RSV-inhibitory concentration and the cytotoxic concentration of each compound. Cultures of the human laryngeal carcinoma cell line HEp-2 and the murine renal cell line MA-104 were established and infected with an MOI=0.005. Cultures were fed twice daily. Treatment with either ribavirin or spA_4-antiRSV3'3'T/(8281-8299) was begun simlutaneously with infection and continued for four days. Treatment was then withdrawn and the test read on day 5. The effects of treatment on RSV infection were reported as 1) an EC_{50}, the concentration at which there was a 50% reduction in the observable cytopathic effects of infection and 2) an EC_{90}, the concentration at which there was a 90% reduction in viral production. The cytotoxic concentration, the IC_{50} was taken as the concentration that resulted in a 50% reduction in cell number. Therapuetic efficacy is estimated by the Selectivity Index, which is the ratio of IC_{50}/EC_{50}.

The results are shown in Tables 5 and 6. Table 5 shows that in HEp-2 cells spA_4-antiRSV3'3'T/(8281-8299) had an EC_{50} of 0.3 μM; ribarvirin had an EC_{50} of 4 μM. The IC_{50}s were >10 μM and 41 μM, respectively. Thus, spA_4-antiRSV3'3'T/(8281-8299) had an SI more than three fold higher than ribavirin.

Table 6 shows the analogous results concerning MA-104 cells. The SI of spA_4-antiRSV3'3'T/(8281-8299) and ribavirin were found to be >500 and about 200, respectively.

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Table 1. Modified Procedure for the Synthesis of Core (2',5')-Oligoadenylate/antisense Chimeras

synthesis region (sequence edit)	coupling time (seconds)	coupling reagents & concentration	trityl mode
oligomer antisense (DNA antisense)	15	0.1 M monomer in acetonitrile	Trityl ON
linker	300	0.1 M linker in acetonitrile	Trityl ON
(2',5')oligoadenylate	600	0.1 M (2',5')Ado^{Bz} in acetonitrile	Triyl ON
Phosphorylation	60	0.1 M P^{III} reagent in acetonitrile	Trityl OFF

Table 2. Synthesis Procedure for 5'-Terminal Phosphorylation

Step	Solvent/reagent	Time	Volume
1. coupling	0.2 M phosphorylation reagent in tetrazole/acetonitrile	3 min.	0.15 mL
2. washing	acetonitrile		3 mL
3. drying	argon	3 min.	
4. oxidation	0.1 M I2 in lutidine:THF:water (20:80:1)	0.75 min.	1 mL
5. washing	acetonitrile		3 mL
6. drying	argon	3 min.	
7. detritylation	3% TCA in CH2Cl2	1.5 min.	1 mL
8. washing	2% Py in acetonitrile		1 mL
9. washing	acetonitrile		3 mL

Table 3. Antiviral activities of chimeric antisense against RSV L polymerase RNA translation start site.

Oligo / (site in RSV RNA)	Structure of Compounds [oligonucleotide] / treatment):	% Inhibition of RSV replication 3.3 μM	6.6 μM	9.9 μM
A_4-antiRSV3'-3'C/(8490-8509)	A2'p(A2'p)$_3$-[(Bu)p]$_2$-(5'aat ggg atc cat ttt gtc c3'-3'c5')		20 (1)	28.3 (3)
pA_4-antiRSV/(8490-8509)	pA2'p(A2'p)$_3$-[(Bu)p]$_2$-(5'aat ggg atc cat ttt gtc cc3')	3 (2)	38.8 (4)	64.8(5)
pA_4-3'antiRSV5'/(8490-8509)	pA2'p(A2'p)$_3$-[(Bu)p]$_2$-(3'ccc tgt ttt acc tag ggt aa5')			69 (1)
pA_4-antiRSV3'-3'C/(8490-8509)	pA2'p(A2'p)$_3$-[(Bu)p]$_2$-(5'aat ggg atc cat ttt gtc c3'-3'c5')	2 (1)	64.3 (3)	74 (2)
spA_4-antiRSV/(8490-8509)	spA2'p(A2'p)$_3$-[(Bu)p]$_2$-(5'aat ggg atc cat ttt gtc cc3')	16.5 (2)	71 (1)	94 (1)

Antiviral activity as determined by virus plaque assay at 36 hrs post-infection. In parentheses, are the number of times the experiments were done to produce the data. Results shown are the average of the percent inhibition data from 2 to 7 experiments, except for single experiment data. Percent inhibition is defined as [(infectious virus particles produced in oligonucleotide treated cells)/(infectious virus particles produced in untreated cells)]X100. M.O.I. was 2.0 p.f.u. per cell.

Chimeric 2-5A-antisense oligonucleotides are abbreviated according to the following convention employed herein for added clarity. The 2-5A domain of the chimera is indicated in bold-face capitalized type; i.e., pA2'p(A2'p)$_3$. The linker moiety is abbreviated as [(Bu)p]$_2$ which stands for two 1,4-butanediol molecules linked through phosphodiester bonds to each other and the 2-5A and antisense domains of the chimera; i.e., -[(Bu)p]$_2$. The antisense oligonucleotide is represented in lower case bold-face type in triplet repeats with normal 3',5' phosphodiester linkages in the polarity shown; i.e., (3'ccc tgt ttt acc tag ggt aa5'). Exceptions include the inversion of polarity for the entire chain or for the terminal 3'-nucleotide, both of which are clearly indicated. When the 5'-monophosphate of the 2-5A domain of the chimera was modified to the 5'-thiophosphate, the abbrevation used is spA2'p(A2'p)$_3$.

Table 4. Antiviral activities of stabilized chimeric antisense against different sites in RSV M2 and L mRNAs.

Oligo / (site in RSV RNA)	Structure of Compounds [oligonucleotide] / treatment):	% Inhibition of RSV replication 3.3 μM	6.6 μM	9.9 μM
spA4-antiRSV3'-3'N Series:				
spA4-antiRSV3'-3'A/(8530-8547)	spA2'p(A2'p)3-[(Bu)p]2-(5'cta tcg gtt aga taa ac3'-3'a5')	2.5 (1)		
spA4-antiRSV3'-3'G/(8599-8618)	spA2'p(A2'p)3-[(Bu)p]2-(5'gat aag gac cat tga ata t3'-3'g5')	44 (1)		
spA4-antiRSV3'-3'C/(8561-8578)	spA2'p(A2'p)3-[(Bu)p]2-(5'ctc tga gaa aga gat aa3'-3'c5')	57 (1)		
spA4-antiRSV3'-3'T/(8261-8279)	spA2'p(A2'p)3-[(Bu)p]2-(5'gat tga aat ata gtg tgt3'-3't5')	78 (2)	87 (1)	87 (1)
spA4-antiRSV3'-'3A/(8251-8270)	spA2'p(A2'p)3-[(Bu)p]2-(5'ata gtg tgt tct ttt gat t3'-3'a5')	86.7 (2)	92 (1)	
spA4-antiRSV3'-3'T/(8281-8299)	spA2'p(A2'p)3-[(Bu)p]2-(5'atg gtt att tgg gtt gtt3'-3't5')	91.3 (3)	97 (1)	99.6 (1)

see legend to Table 3.

Table 5.

ANTIVIRAL ACTIVITY of spA4-antiRSV3'-3'T /(8281)

Hep-2 Cells

Neutral Red

Compound	EC_{50}	IC_{50}	SI
spA4-antiRSV3'-3'T /(8281)	0.3 μM	>10 μM	>33
Ribavirin	4 μM	41 μM	10

determined with RSV strain A2, MOI = 0.005

Fresh medium and oligo or ribavirin added twice daily for 4 d and test read on d 5.

EC_{50} effective concentration to reduce RSV-induced CPE by 50%.

IC_{50} 50% inhibitory concentration for cytotoxicity to cells (visual and dye uptake as determined in rapidly dividing cells as opposed to stationary cells used for viral assays.

SI selectivity index = IC_{50}/EC_{50}

Table 6.

ANTIVIRAL ACTIVITY of spA4-antiRSV3'-3'T /(8281)

MA-104 Cells

Visual CPE and Virus Yield Reduction

Compound	EC_{50}	EC_{90}	IC_{50}	SI
spA4-antiRSV3'-3'T /(8281)	0.02 μM	0.02 μM	>10 μM	>500
Ribavirin	1 μM	7 μM	210 μM	210

as determined with RSV strain A2, MOI = 0.005

Fresh medium and oligo or ribavirin added twice daily for 4 d and test read on d 5.

EC_{50} effective concentration to reduce RSV-induced CPE by 50%.

EC_{90} effective concentration to reduce RSV yield by 90%.

IC_{50} 50% inhibitory concentration for cytotoxicity to cells (visual and dye uptake as determined in rapidly dividing cells as opposed to station ary cells used for viral assays.)

SI selectivity index = IC_{50}/EC_{50}

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Cleveland Clinic Foundation and
National Institutes of Health

(ii) TITLE OF THE INVENTION: RNase L Activators and
Antisense Oligonucleotides Effective to
Treat RSV Infections

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: NY
(E) COUNTRY: U.S.A.
(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: unfiled
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/011,725
(B) FILING DATE: 2/15/96

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Poissant, Brian M.
(B) REGISTRATION NUMBER: 28,462
(C) REFERENCE/DOCKET NUMBER: 8656-009-228

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-790-9090
(B) TELEFAX: 212-869-9741
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: RSV (L+)
(B) LOCATION: 1...19
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCAATGGTCC TTATCTCAA 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: RSV (L-)
(B) LOCATION: 1...19
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAGCTTTATT AGCAGCATC 19

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: GAPDH (+)
(B) LOCATION: 1...19
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAATCCCATC ACCATCTTC 19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: GAPDH (-)
(B) LOCATION: 1...19
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CACCACCCTG TTGCTGTAG 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: RSV (M2+)
(B) LOCATION: 1...19
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAACAATCAG CATGTGTTG 19

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: RSV (M2-)
(B) LOCATION: 1...19
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATGTAACGA TGTGGTGAG 19

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Activator-antisense complex
(B) LOCATION: 1...19
(D) OTHER INFORMATION: spA4-antiRSV3'-3'T/(1-19)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTGTACGCAT TTTTTCGCG 19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Activator-antisense complex
(B) LOCATION: 1...19
(D) OTHER INFORMATION: spA4-antiRSV3'-3'T/(51-69)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTACTTATCA AATTCTTAT 19

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Activator-antisense complex
(B) LOCATION: 1...18
(D) OTHER INFORMATION: spA4-antiRSVGe3'-3'T/(1-18)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACGCGAAAAA ATGCGTAC 18

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Activator-antisense complex
(B) LOCATION: 1...18
(D) OTHER INFORMATION: spA4-antiRSVGe3'-3'T/(84-101)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTCCCTTGGT TAGAGATG 18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Activator-antisense complex
(B) LOCATION: 1...18
(D) OTHER INFORMATION: spA4-antiRSVGe3'T/(369-386)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAAATGATGG AATTAACA 18

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...19
(D) OTHER INFORMATION: A4-antiRSV3'-3C/(8490-8509)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATGGGATCC ATTTTGTCC 19

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...20
(D) OTHER INFORMATION: pA4-antiRSV/(8490-8509)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATGGGATCC ATTTTGTCCC 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...20
(D) OTHER INFORMATION: pA4-3'antiRSV5'/(8490-8509)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AATGGGATCC ATTTTGTCCC 20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...19
(D) OTHER INFORMATION: pA4-antiRSV3'-3'C/(8490-8509)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATGGGATCC ATTTTGTCC 19

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...20
(D) OTHER INFORMATION: spA4-antiRSV/(8490-8509)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATGGGATCC ATTTTGTCCC 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...17
(D) OTHER INFORMATION: spA4-antiRSV3'-3'A/(8530-8547)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTATCGGTTA GATAAAC 17

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...19
(D) OTHER INFORMATION: spA4-antiRSV3'-3'G/(8599-8618)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GATAAGGACC ATTGAATAT 19

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...17
(D) OTHER INFORMATION: spA4-antiRSV3'-3'C/(8561-8578)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTCTGAGAAA GAGATAA 17

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...18
(D) OTHER INFORMATION: spA4-antiRSV3'-3'T/(8261-8279)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GATTGAAATA TAGTGTGT 18

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 1...19
(D) OTHER INFORMATION: Oligonucleotide spA4-anti RSV3'-3'A/(8251-8270)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATAGTGTGTT CTTTTGATT 19

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide
(B) LOCATION: 1...18
(D) OTHER INFORMATION: spA4-antiRSV3'-3'T/(8281-8299)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGTTATTT GGGTTGTT 18

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA
(ix) FEATURE:

(A) NAME/KEY: RSV-A2
(B) LOCATION: 1...15222
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CATAGATTAT CATCCAACAC ATATGAAAGC AATATTAACT TATATAGATC TTGTTAGAAT  13440
GGGATTGATA AATATAGATA GAATACACAT TAAAAATAAA CACAAATTCA ATGATGAATT  13500
TTATACTTCT AATCTCTTCT ACATTAATTA TAACTTCTCA GATAATACTC ATCTATTAAC  13560
TAAACATATA AGGATTGCTA ATTCTGAATT AGAAAATAAT TACAACAAAT TATATCATCC  13620
TACACCAGAA ACCCTAGAGA ATATACTAGC CAATCCGATT AAAAGTAATG ACAAAAAGAC  13680
ACTGAATGAC TATTGTATAG GTAAAAATGT TGACTCAATA ATGTTACCAT TGTTATCTAA  13740
TAAGAAGCTT ATTAAATCGT CTGCAATGAT TAGAACCAAT TACAGCAAAC AAGATTTGTA  13800
TAATTTATTC CCTATGGTTG TGATTGATAG AATTATAGAT CATTCAGGCA ATACAGCCAA  13860
ATCCAACCAA CTTTACACTA CTACTTCCCA CCAAATATCT TTAGTGCACA ATAGCACATC  13920
ACTTTACTGC ATGCTTCCTT GGCATCATAT TAATAGATTC AATTTTGTAT TTAGTTCTAC  13980
AGGTTGTAAA ATTAGTATAG AGTATATTTT AAAAGATCTT AAAATTAAAG ATCCCAATTG  14040
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TATAGCATTC ATAGGTGAAG GAGCAGGGAA TTTATTATTG CGTACAGTAG TGGAACTTCA  14100
TCCTGACATA AGATATATTT ACAGAAGTCT GAAAGATTGC AATGATCATA GTTTACCTAT  14160
TGAGTTTTTA AGGCTGTACA ATGGACATAT CAACATTGAT TATGGTGAAA ATTTGACCAT  14220
TCCTGCTACA GATGCAACCA ACAACATTCA TTGGTCTTAT TTACATATAA AGTTTGCTGA  14280
ACCTATCAGT CTTTTTGTCT GTGATGCCGA ATTGTCTGTA ACAGTCAACT GGAGTAAAAT  14340
TATAATAGAA TGGAGCAAGC ATGTAAGAAA GTGCAAGTAC TGTTCCTCAG TTAATAAATG  14400
TATGTTAATA GTAAAATATC ATGCTCAAGA TGATATTGAT TTCAAATTAG ACAATATAAC  14460
TATATTAAAA ACTTATGTAT GCTTAGGCAG TAAGTTAAAG GGATCGGAGG TTTACTTAGT  14520
CCTTACAATA GGTCCTGCGA ATATATTCCC AGTATTTAAT GTAGTACAAA ATGCTAAATT  14580
GATACTATCA AGAACCAAAA ATTTCATCAT GCCTAAGAAA GCTGATAAAG AGTCTATTGA  14640
TGCAAATATT AAAAGTTTGA TACCCTTTCT TTGTTACCCT ATAACAAAAA AAGGAATTAA  14700
TACTGCATTG TCAAAACTAA AGAGTGTTGT TAGTGGAGAT ATACTATCAT ATTCTATAGC  14760
TGGACGTAAT GAAGTTTTCA GCAATAAACT TATAAATCAT AAGCATATGA ACATCTTAAA  14820
ATGGTTCAAT CATGTTTTAA ATTTCAGATC AACAGAACTA AACTATAACC ATTTATATAT  14880
GGTAGAATCT ACATATCCTT ACCTAAGTGA ATTGTTAAAC AGCTTGACAA CCAATGAACT  14940
TAAAAAACTG ATTAAAATCA CAGGTAGTCT GTTATACAAC TTTCATAATG AATAATGAAT  15000
AAAGATCTTA TAATAAAAAT TCCCATAGCT ATACACTAAC ACTGTATTCA ATTATAGTTA  15060
TTAAAAATTA AAAATCATAT AATTTTTTAA ATAACTTTTA GTGAACTAAT CCTAAAGTTA  15120
TCATTTTAAT CTTGGAGGAA TAAATTTAAA CCCTAATCTA ATTGGTTTAT ATGTGTATTA  15180
ACTAAATTAC GAGATATTAG TTTTTGACAC TTTTTTTCTC GT                      15222
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WE CLAIM:

1. A composition comprising a polynucleotide consisting essentially of:

a) an antisense oligonucleotide, having a hydroxyl moiety at a first end, in which oligonucleotide is complementary to between 15 and 20 nucleotides of the antigenomic RNA strand of a strain of a Respiratory Syncytial Virus;

b) a linker attached to the first end; and

c) an oligonucleotide activator of RNase L attached to the linker.

2. The composition of claim 1, in which the antisense oligonucleotide is complementary to at least 15 contiguous nucleotides of the antigenomic RNA strand that are normally single stranded.

3. The composition of claim 1, in which the antisense oligonucleotide is complementary to a portion of a region of the antigenomic RNA strand that is normally single stranded, said region having greater than 34 nucleotides.

4. The composition of claim 1, in which the antisense oligonucleotide is complementary to a portion of a region of the antigenomic RNA strand that is normally single stranded, said region having greater than 45 nucleotides.

5. The composition of claim 1 in which the oligonucleotide activator is selected from the group consisting of sp5'A2'(p5'A2')$_2$-O-, sp5'A2'(p5'A2')$_3$-O-, p5'A2'(p5'A2')$_2$-O-, and p5'A2'(p5'A2')$_3$-O-.

6. The composition of claim 1 in which the first end is the 5' terminus, and the 3' terminal hydroxyl of the antisense oligonucleotide is blocked by a blocker selected from the group consisting of a -p3'N5' nucleotide, a p-O-alkylamine, a p-O-

hydroxyalkylamine, a sp-O-alkylamine, a sp-O-hydroxyalkylamine, ethyl and methyl.

7. The composition of claim 1 in which the first end is the 3' terminus.

8. The composition of claim 1 in which the strain Respiratory Syncytial Virus is the A2 strain, and the portion of the antigenome is between residues 617-663 or residues 718-772, numbered in the 5'→3' direction.

9. The composition of claim 1 in which the strain Respiratory Syncytial Virus is the A2 strain, and the portion of the antigenome is between residues 2490 and 2530, numbered in the 5'→3' direction.

10. The composition of claim 1 in which the strain Respiratory Syncytial Virus is the A2 strain, and the portion of the antigenome is between residues 8251 and 8299, numbered in the 5'→3' direction.

11. The composition of claim 10, in which the antisense oligonucleotide is selected from the group consisting of residues 8251-8270, 8261-8279 and 8281-8299, numbered in the 5'→3' direction.

12. The composition of claim 1, in which the antisense oligonucleotide contains one or more phospho-moieties selected from the group consisting of phosphorothioate, methylphosphonate and methylphosphonothioate.

13. The composition of claim 1, in which the antisense oligonucleotide contains one or more 2'O-methyl nucleotides.

14. A composition which comprises an effective concentration of the polynucleotide of claim 1 and a pharmaceutically acceptable carrier.

15. The composition of claim 14, which comprises a pharmaceutically acceptable, aerosolizable carrier.

16. A method of treatment which comprises a step of forming in a subject having a Respiratory Syncytial Virus infection an effective amount of a complex comprising:

a) an antisense oligonucleotide, in which the sequence of said oligonucleotide is complementary to between 15 and 20 nucleotides of a normally single stranded portion of the antigenomic RNA strand of a strain of a Respiratory Syncytial Virus; and

b) an activated RNase L.

17. A method of treatment which comprises a step of administering to a subject having a Respiratory Syncytial Virus infection an effective amount of a composition comprising a polynucleotide consisting essentially of:

a) an antisense oligonucleotide, having a hydroxyl moiety at a first end, in which the sequence of said oligonucleotide is complementary to between about 15 and 20 nucleotides of a normally single stranded portion of the antigenomic RNA strand of a strain of a Respiratory Syncytial Virus;

b) a linker attached to the first end;

c) an activator of RNase L attached to the linker; and

d) a pharmaceutically acceptable, aerosolizable carrier.

18. A compound comprising an antisense oligonucleotide of 15 to 20 5'-3'-linked nucleotides, which oligonucleotide is complementary to a portion of a RSV antigenomic RNA strand selected from the group consisting of residues 617-663, residues 718-772, residues 2490-2530, residues 3212-3247, residues 5240-5288, and residues 8251-8299 of the RSV strain A2 genome, numbered in the 5'→3' direction.

19. The compound of claim 18 in which the antisense oligonucleotide is 18 or 19 5'-3'-linked nucleotides.

```
   1 acgagaaaaa aagtgtcaaa aactaatatc tcgtaattta gttaatacac
  51 atataaacca attagattag ggtttaaatt tattcctcca agattaaaat
 101 gataacttta ggattagttc actaaaagtt atttaaaaaa ttatatgatt
 151 tttaattttt aataactata attgaataca gtgttagtgt atagctatgg
 201 gaatttttat tataagatcc ttattcatta ttcattatga aagttgtata
 251 acagactacc tgtgatttta atcagttttt taagttcatt ggttgtcaag
 301 ctgtttaaca attcacttag gtaaggatat gtagattcta ccatatataa
 351 atggttatag tttagttctg ttgatctgaa atttaaaaca tgattgaacc
 401 attttaagat gttcatatgc ttatgattta taagtttatt gctgaaaact
 451 tcattacgtc cagctataga atatgatagt atatctccac taacaacact
 501 ctttagtttt gacaatgcag tattaattcc tttttttgtt atagggtaac
 551 aaagaaaggg tatcaaactt ttaatatttg catcaataga ctctttatca
 601 gctttcttag gcatgatgaa atttttggtt cttgatagta tcaatttagc
 651 attttgtact acattaaata ctgggaatat attcgcagga cctattgtaa
 701 ggactaagta aacctccgat ccctttaact tactgcctaa gcatacataa
 751 gtttttaata tagttatatt gtctaatttg aaatcaatat catcttgagc
 801 atgatatttt actattaaca tacatttatt aactgaggaa cagtacttgc
 851 actttcttac atgcttgctc cattctatta gttggttgca tctgtagcag
 901 gttacagaca attcggcatc acagacaaaa agactgatag gttcagcaaa
 951 ctttatatgt aaataagacc aatgaatgtt gttggttgca tctgtagcag
1001 gaatggtcaa attttcacca taatcaatgt tgatatgtcc attgtacagc
1051 cttaaaaact caataggtaa actatgatca ttgcaatctt tcagacttct
1101 gtaaatatat cttatgtcag gatgaagttc cactactgta cgcaataata
1151 aattccctgc tccttcacct atgaatgcta tacaattggg atctttaatt
1201 ttaagatctt ttaaaatata ctctatacta attttacaac ctgtagaact
1251 aaatacaaaa ttgaatctat taatatgatg ccaaggaagc atgcagtaaa
1301 gtgatgtgct attgtgcact aaagatattt ggtgggaagt agtagtgtaa
1351 agttggttgg atttggctgt attgcctgaa tgatctataa ttctatcaag
1401 cacaaccata gggaataaat tatacaaatc ttgtttgctg taattggttc
1451 taatcattgc agacgattta ataagcttct tattagataa caatggtaac
1501 attattgagt caacattttt acctatacaa tagtcattca gtgtcttttt
1551 gtcattactt ttaatcggat tggctagtat attctctagg gtttctggtg
1601 taggatgata taatttgttg taattatttt ctaattcaga attagcaatc
1651 cttatatgtt tagttaatag atgagtatta tctgagaagt tataattaat
1701 gcagaagaga ttagaagtat aaaattcatc attgaatttg tgtttatttt
1751 taatgtgtat tctatctata tttatcaatc ccattctaac aagatctata
1801 taagttaata ttgctttcat atgtgttgga tgataatcta tgttaacaac
1851 ccaagggcaa actgtgaatt ctgctacatt aagacgttta agaaaccata
1901 atttgaagct atgacatcct tttactctat gtaaacttgc atcttggcta
1951 agaatgtatt tgataacttt ttgttctaaa aataccttag acatagactt
2001 ccaataacta ctgtctatta attccaatac acatagaaga tctgaagtgt
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FIG.1A

2051 tcatatcaca ctccagcttt gctttgccat aacctttatg aaaacacaag
2101 agataggtct tataagcatt gaagaaaact ttcaaattaa taaacatatg
2151 atcagttata tatccctctc cccaatcttt ttcaaaaata cctttagaat
2201 ctttcataag ttgtataatc agaatccaat gtccagctaa attagtactt
2251 aaaatgtaag tattatgaaa atagtcagat attttatgtg ccaatattaa
2301 attagaatta acatgagatc cagatttgag tgttttatta cttaagaata
2351 attccacata ttgagtcaaa cttattttgt ctggtaaaaa catatgctgt
2401 ttttgtatca cttgttttaa cttgtgaata tcaacatcac ctgtgaatat
2451 gggaggtttc atcaaatgta tctcattaag cttaggtatg agaataattc
2501 tgttaggaca tacattagta aattgttcta ctactgacat taaactaagg
2551 ccaaagctta tacagttttg gaatactatg tcaatatctt catcaccata
2601 cttttctgtt aatatgcgat taatagggct agtgtcaaag tgataatttg
2651 ttgttctata agctggtatt gatgcaggga attcacatgg tctactactg
2701 actgtaaggc gatgcaaata attgacactt aaatattgtg gaaataattt
2751 cttggccttt tcatatgtta acccaagggt tcctatgctg agttcttcca
2801 tgaattcatc cttgttatct atagatgcat acacccaatc caattttgct
2851 aatagatcta tttgatctct ctgtttttg gttaagactt gtctattata
2901 aactaacatt atttttttct cttgtgtaga tgaaccaacc catggtttag
2951 tgggtcctct ctcaccacgt gttaaactgt taacattata tttctctata
3001 attatgccac tagatatagt gcttgtagta tatttgatgt ccattgtata
3051 catgatactg ggtgatgtaa caccaactat attggataaa gaccaagatc
3101 tttccctaac atatttgctt aattcagtaa tacttaggtt ttccatactc
3151 aatatctctc ttttatctct gttacaatcc aatggaagta tccttataag
3201 caaagttatg tttttcctca tcatctcagt ggctctatca atatctgtta
3251 agtctatggc agaagttttt tccagtatgt tagttataga ttttgtacct
3301 gatataagat ttactatttt ctctgcttta taaaagggta aactttcata
3351 aacaactctt agcccatgag gatatgtagg ttctatattt tgcataatat
3401 catttagatc tatctctgta gtagtataat gttgtgcact tttggagaat
3451 attttgtttg gagctgtact caaaacctct gtaactgcca gtctattgat
3501 ttcgctagta attttagctt gtctctcaga ccctaaagct tgaggatctc
3551 tcatcaatgt tacgaattca gcattagggt ttttgtcaaa cgtgattatg
3601 catgttaaga acttattcaa tctatcatct gacagatctt gaagtttatc
3651 ttttaagtca tggtttgtat aataactaag tatgaacaca gagtgaacta
3701 tagcctctgt gaggaagtca ggagttcttc tatagaaact tcgatataac
3751 aagttgggat caccaccacc aaataacatg ggtaaattca tatacaatgt
3801 taatgctgta tcaatattat caagattaaa aaaggttttt aagtgtttca
3851 gaacctttaa tatgtccaaa tatagtttat tgttacataa tgcatgattt
3901 tttaattgta gagcaatctg attatataac catacatttc taaatattaa
3951 actgcataat agactttcac ctctatattc taattcttgt gtcaaactac
4001 ctatagattc tagactcact ttgaaatcat caagtatagt gtttatccac
4051 ggtcccactc ttaggacttt ctttatacta gctgggtaat atacaccgtt
4101 atgttgaatt gttttactca taaattgcat atctcgtgat atataagtct

FIG. 1B

4151 cagttccttt taatttgtgg cctatgcctg catactcttt atacagtaat
4201 ttaaggctat ttaatgctag caaataatct gcttgagcat gagtttgacc
4251 ttccatgagt ctgattggtt tgcttatatc tattgattga ttgtcaccat
4301 taattaaagc agtaattgag aatttccctt tgagagatat tagatccaat
4351 agtgatatag cttctatggt ccacagtttt tgacaccacc cttcgatgcc
4401 acccatgtga tatctatata atccactttg ttcatctaca ttgttaagat
4451 ctacaatatg atctcctata taggggggtg catgcctata tgtgcatatt
4501 attgtgacat gaggaatagt taaatgtaac caggaaaata gagattgtac
4551 accatgcagt tcatccagca catcactaca aatacatgac gtttcatatc
4601 gaaatgcttg attgaatttg ctgagatctg tgatgataga gcacttacta
4651 atgtaattgt tgtaattatc attgtagcga tttgatttgt tacttattcc
4701 tgctttcaat tctaatattt tttgtagttc tagatcacca tatcttgtaa
4751 gactttcagg aaagaattgt aaaatgtttt cagctatcat tttctctgcc
4801 aatatttgaa cctgtctgaa cattcccggt tgcattgcaa acattctacc
4851 tacactgagt tctctttctt tgcctgtcaa tgataccaca tgattagggt
4901 tgttgagata actttgatta actacacagt tgtataaatc acattcattg
4951 aatttgttat ctcttaaata atactctaat actcttcttg atttatcact
5001 ctcggaaaat tttaattttt catgttctat atagttttgt atgtgtgatg
5051 gcatgtaatt tctagggaaa ctagtccata tcaaattttt aggaggtgat
5101 atagctttat catttataat catttcaaga tccacttttt taggcaaccg
5151 aaactcacga tagaaacgta gtcctgataa cacaatcaaa tctctttctg
5201 taagttccaa caaagaagga taagtgttta gtttatagta agttaaccat
5251 cttaagggta aaacaatagc atttcttaaa gtaggccatc tgttgtaatt
5301 atttacaaac ccttttataa ttctatatat aaaggcacct cttaacatac
5351 tcagactgct taacaagtaa aatttggtct cattgcaatt aattttaaca
5401 gcatccatgg cttgtctttc atctaccatt gggtgtccaa atattctgaa
5451 caaaaaatat agttcactca gattgttaag gttattgtca cctgcaagct
5501 taattaattt aaggaactta cttaatagaa ttatccatct gccatttatt
5551 atattatcgg acactgtctt atctaataat gtatgacata ctcttgatag
5601 cagatttttc tgagctttat tagcagcatc tgtgatgttg ttgagcatac
5651 tattataaaa tcgttttctg aattgatctt cttctgttat atttaaaatt
5701 agagacataa taaatccctc tacctctttt attatgtaga acccctcatt
5751 gtgaaatagc tttagtatac aatctccata aaggaatagt tgtgtcaaga
5801 taacattatt gaatccgcat cttaagccta agcttttatt taatgtgttc
5851 aagcagttac taatccatgt aattaaacaa acatttaatc tactaaggct
5901 aatatctttc catgtcaaga attgattata ggttgtcaca gtaattcttt
5951 tgagttcctt atgataaact atacaaccat attggttcaa aataaattga
6001 aatccactaa gagtttgatt atctatcaat gtaaacccat ggttttttac
6051 ctcatttgat cgatactgtg ttaatatgtt gtttaatttt gtgtataagt
6101 taaaccaatg tattaaccat gatggaggat gttgcattga acacatcaat
6151 ttcttcaaga gtgttgtttt gattgtgtct ttttgttttg tagagtgatt
6201 tttgtctgct ttaagatgag attgattatc tttaacagct gaaagtatat

FIG. 1C

6251 tttgtctgct tatggtcgta ataactgagt tgtcttcatc ttgtccattg
6301 ttggatttaa tcttgtcctt ttctttaagt tgtcttcatc ttgtccattg
6351 atagactttg acatcactta tttctatagc tcttcttatt atctttttaa
6401 gtaaattagt ggtagcaatc tgttctgacg aggtcatact cttgtatgtc
6451 ataagtaatg actgaaaata agtaggttct tctaatttta tttcaccttt
6501 atgatactta gatattaagg actgtgttat atttagtttc tttagattca
6551 tgtgttctat taatggattt tgtctactaa ttaagttggt ataatcattt
6601 ttgagataag gaccattgaa tatgtaactt cctaaagcat tacactctga
6651 gaaagagata acacctttta aataactatc ggttagataa acattagcag
6701 aatttccatt aataatggga tccattttgt cccacaactt gaattgtttg
6751 aattaataat gtaacgatgt ggtgagtgtt agaattgagt gttatgacac
6801 taatatatat attgtatata tatcctcaat aatacctaga tgttgtagaa
6851 aaatttgaat tgtgtcaatc aattcttgag aggtccaatg gatttcattg
6901 aatgtttgat tcggtgagta catatggtta tttgggttgt tttgattgaa
6951 atatagtgtg ttcttttgat tatacatagt aactctacat ctacttgtta
7001 ttagtatgga agttatacta caaggatatt tgtcaggtag tatcattatt
7051 tttggcatgg tcatttgtat cactaacagt tgattctttt gggttgttga
7101 tggttatgct cttatggata tccaatgtgt ttttgatggt tttcttcaat
7151 acgtctgctg gcaatctttt taacagatgg atagtttgtt tattgttttt
7201 cctgttgctt tcaatatatg atatgacagt attgtacact cttatcttgg
7251 gtgaatttag ctcttcattg tccctcagct ttttgatatc atcactattg
7301 agttcagtga ggagtttgct catggcaaca catgctgatt gtttagttat
7351 attgtttatt gatcctatat aactctctag cactccaact acaccaagag
7401 catactcttc tgttctgtcc aactctgcag ctccacttat ttctgataag
7451 gtatctatac ttttatccat agacttaagt attctgttta acataaagtt
7501 ttgtcttaca agcagtgcat ggggtggcca ttcaaaataa ttatgactaa
7551 aatgacacct cttaccattt aagcaatgac ctcgaatttc aaatttgcaa
7601 ggattccttc gtgacatatt tgccccagtt ttcattttta cagatggtaa
7651 gttaatctgg cattcaattg tgttttatat aactataaac taggaatcta
7701 cttaaatagt gtaagtgaga tggtttatag atgagagttt cgatgaagtt
7751 cagattttaa gaaaatccaa tgacagatgg gttgtctatg agcagatagt
7801 aaaccattgt aagaacatga ttaggtgcta ttttatttta gttactaaat
7851 gcaatattat ttataccact cagttgatct ttgcttagtg tgactggtgt
7901 gcttctggcc ttacagtata agagcagtcc aacagcaatt aatgataaca
7951 atattactat aatcactata attatagtag ttatcatgat atttgtggtg
8001 gatttaccag catttacatt atgtaataat tcatcggatt tacgaataaa
8051 tgctaggctc tggttaatct tctcgttgac ttgagatatt gatgcatcaa
8101 attcatcaga ggggaatact aatgggtcat agaaatttat tattggttca
8151 ccttttacat agagactttt accttcttgc ttatttacat aatataatgt
8201 gttacctaca gacacagtgt ccatcccttt atttgataca taatcgcacc
8251 cgttagaaaa tgtctttatg attccacgat ttttattgga tgctgtacat
8301 ttagttttgc catagcatga cacaatggct cctagagatg tgataacgga

FIG. 1D

8351 gctgcttaca tctgtttttg aagtcataat tttacaatca tatttggggt
8401 tgaatatgtc aacattgcag agatttattt cacttggtaa tgttaaactg
8451 ttcattgtgt cacaaaatac tcgatttgat tgaactttac atgtttcagc
8501 ttgtgggaag aaagatactg atcctgcatt gtcacagtac catcctctgt
8551 cagttcttgt taaacagatg ttggaccctt cttttgtgtt ggttgtacat
8601 agaggggatg tgtgtagttt ccaacagggt gtatctataa caccatatag
8651 tggtaattgt actacatatg ctaagacttc ctcttttatt atggacatga
8701 tagagtaact ttgctgtcta actatttgaa cattgttgga cattaacttt
8751 ttctgatcat ttgttatagg catatcattg attaatgaca ataattcact
8801 attagttaac atgtaagtgc ttacaggtgt agttacacct gcattaacac
8851 taaattccct ggtaatctct agtagtctgt tgttcttttg ttggaactct
8901 atcacagttt ctatatttga tatgctgcag ctttgcttgt tcacaatagg
8951 taacaattgt ttatctatat agtttttgag gtctaacact ttgctggtta
9001 agacactaac tccatttgat aagctgacta gagccttgtt tgtggatagt
9051 agagcacttt tgatcttgtt cacttcccct tctaggtgca ggaccttaga
9101 tacagcaacg ccactggcga ttgcagatcc aacacctaac aaaaaaacaa
9151 gaaatcttct tttccttttc ttgcttaatg ttacattggt ttttttggca
9201 ttgttgagtg tataattcat aaaccttggt agttctcttc tggctcgatt
9251 gtttgttggt ggtgtgcttt gcatgagcaa ctgcaattct gttacagcat
9301 ttttatattt atctaattct tgttttatca attttacctt agcatctgtt
9351 ccattacact tattttcctt gatattactt aattctatag ttataacact
9401 ggtataccaa ccagttctca gagcactaag atagcctttg ctaactgcac
9451 tgcatgttga ttgataaaat tcttcagtga tgttttgacc agaagcaaaa
9501 caaaatgtga ctgcagtgag gattgtggta attgcatttg ctttgaggat
9551 tagcaactcc attgttattt gccccagagt ttattttgat tctgtttaag
10001 tggtggtggg tatgctgcag ggtacaaagt tggcgttgtt aaagtgaaaa
10051 tcattattgg gtttgcttgg tggtttgttt tggcgttgtt ttgtggtggg
10101 cttgctgggt tgtgtttgag ttgttgttgt gtttttggtc ttgactgttg
10151 tggattgcag ggttgacttg actcctggtg ttgttgaagc tagtatggtg
10201 gtgatttgtg atgtaatttc agacggatta gagggactga ttccaagctg
10251 aggattctgg gtgaggtatg ttggggttgt gttcttgatc tggcttgttg
10301 catcttgtat gattgcagtt gttggtgtga ctttgtggtt tgccgaggct
10351 atgaatatga tggctgcaat tataagtgaa gttgagatta tcattgccag
10401 aatggataat gtgatttgtg ctacagattt aagatttaac ttatataagc
10451 acgatgatat gaataataaa tgattgagag tgtcccaggt cctttctaat
10501 gtcttagcgg tgcgttggtc cttgtttttg gacatgtttg catttgcccc
10551 aatgttattg ttagtcttga tatcctagtt cattgttatg actattttta
10601 attaactact ttatagtatg gatagtggtt tgcatggtgg gatgttaatg
10651 aggtgttgta aagaggtagg ggttgttcat ttttaaatgc aaggttactg
10701 ttttgggctg ttggattgat gaatgctatg tgttgactcg agctcttggt
10751 aactcaaagg ttttgttatg gaatacgtta tattcacaaa gtttgtttag
10801 tattgcaatc atgatggaga ttatgattag caaagagatt attgttgtga

FIG. 1E

10851 tcatgtgtat tagtgtaaag taaggccaga atttgcttga gaattctatt
10901 gttatggatg tattttccat tggttgattc tgtatggtgt gtggacttgt
10951 ctatgttaac agatattgtg attagttgga tttcctccaa tgattatttg
11001 gaaccacaga atatttttta ttaacttatt tgagtactag atctgataaa
11051 caatgacttg ggatgatctg ggacttcaga taagttttgt ttgattggtt
11101 gaaccacaga gtgtttgtga ttgtgatggt gaagtgaaga atgtaggtag
11151 aaagtttgta tgaattaaca cagtgatgta gaggaaaaag gttaatcttc
11201 catgggtttg attgcaaatc gtgtagctgt gtgcttccaa tttgtggtaa
11251 cataatatat actttctttt tctaggtaag ctccaagatc tactatgaat
11301 tgactttgtg gctttatgta tttgaatgct cctttgttgt cagtcactgt
11351 gatgactaat agtaatcctg agtaagggat gatttttgca tttgtgatag
11401 catttttgaa ttcagtggtt gttatatttt caagtgtgtt cagatcttta
11451 tttctgacac tgatggatct taggtatgtt ggtattatga ctttttttga
11501 tgttactatg ttttcaaatt cacataaagc aataatatca tgtgtagggt
11551 tgagtgtctt catagtgaga tctttaactg tagtcaacat atttttgat
11601 tttaggcatg ttagactaca tgccttgatt tcacagggtg tggttacatc
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12151 atcagaaatc ttcaagtgat agatcattgt cactatcatt cccttccaat
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12251 gtcttttgcc atcttttcac tttcctcatt cctgagtctt gccatagctt
12301 ctaatctgtc attggtcatt aatgcttcag ttctgatttt ttctatcatt
12351 tcttctctta aaccaatcat ggcatctctt ataccatccc gagcagatgt
12401 aggtcctgca cttgccacta ctaatgtgtg aagcattcct agtatttcac
12451 ttaatttttc atcaatccta tctaatcttg ctgttatatt atcgtttgtc
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12651 ttgttccctg cagtatcatc tgtctcattt gttgggttga taatagttga
12701 atttgatgtt atagggcttt ctttggttac ttctatatct attgagttga
12801 ccctttattg attctaggaa tttagtagcc ctgttgtttg catcttctcc
12851 atggaattca ggagcaaact tttccatgat gatttatttg ccccattttt
12901 tattaactca aagctctaca tcattatctt ttggattaag ctgatgtttg
12951 atagcctcta gttcttctgc tgtcaagtct agtacactgt agttaatcac

FIG.1F

13001 accattttct ttgagttgtt cagcatatgc ctttgctgca tcatatagat
13051 cttgattcct cggtgtacct ctgtactctc ccattatgcc taggccagca
13101 gcattgccta atactacact ggagaagtga ggaaattgag tcaaagataa
13151 taatgatgct tttgggttgt tcaatatatg gtagaatcct gcttcaccac
13201 ccaatttttg ggcatattca taaacctcaa caacttgttc catttctgct
13251 tgcacactag catgtcctaa cataatattt ttaactgatt ttgctaagac
13301 tccccaccgt aacatcactt gccctgcacc ataggcattc ataaacaatc
13351 ctgcaaaaat cccttcaact ctactgccac ctctggtaga agattgtgct
13401 ataccaaaat gaacaaaaac atctataaag tggggatgtt tttcaaacac
13451 ttcatagaag ctgttggcta tgtccttggg tagtaagcct ttgtaacgtt
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13601 taatattatc atcccacaat caggagagtc atgcctgtat tctggagcta
13651 cctctcccat ttcttttagc atttttttgt aggattttct agattctatc
13701 tcaatgttga tttgaatttc agttgttaag cttgccaatg ttaacacttc
13751 aaatttcatt tcttttccat taatgtcttg acgatgtgtt gttacatcta
13801 ctccatttgc ttttacatga tatcccgcat ctctgagtat ttttatggtg
13851 tcttctcttc ctaacctaga catcgcatat aacataccta ttaacccagt
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13951 tgatgtgttt ctgcacatca taattaggag tatcaatact atctcctgtg
14001 ctccgttgga tggtgtattt gctggatgac agaagttgat ctttgttgag
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14301 gattgatgaa tattggcata gggaaagtgc catattttgt gttgtattca
14351 gtatattttt tatatttagt gcttcctact ttgtgtaata gtttcatttc
14401 atagttgacc aggaatgtaa atgtggcctg tttttcatca agttttctca
14451 ctatgcattc atgatttatc aagtatataa atttgtgtgt tatgatgtct
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14601 ggttggctga attgatttat ttgccccatt tttgtcttct gttaagtttt
14651 atattaacta atggtgttag tgacattgat ttgctagttg atattaatta
14701 taatttatgg attaagatca aatccaagta attcagataa ttgattcata
14751 taattggtca ttgttgaatc acttagtttt ttggagaatt taatttcaca
14801 attgtcatct agtagaccat taggttgaga gcaatgtgtt aattccatca
14851 tttcccatat ataacctcca ttttgtagta ctggcattgt tgtgaaattg
14901 gattttacta caatattatt attagggcaa atatcactac ttgtaataac

FIG.1G

```
14951  atgcacaaac acaatgccat tcaatttgat tgtatgtatc actgccttag
15001  ccaaagcgtt agttaaatgt attaatttat cagtatagca tgttatttt
15051  aacaatgcta cttcatcatt gtcaaacaaa ttttgtaatc taacttttat
15101  catactcaat gaattgctgc ccatctctaa ccaagggagt taaatttaag
15151  tggtacttat caaattctta tttgccccat ttttttggtt tatgcaagtt
15201  tgttgtacgc atttttttcgc gt
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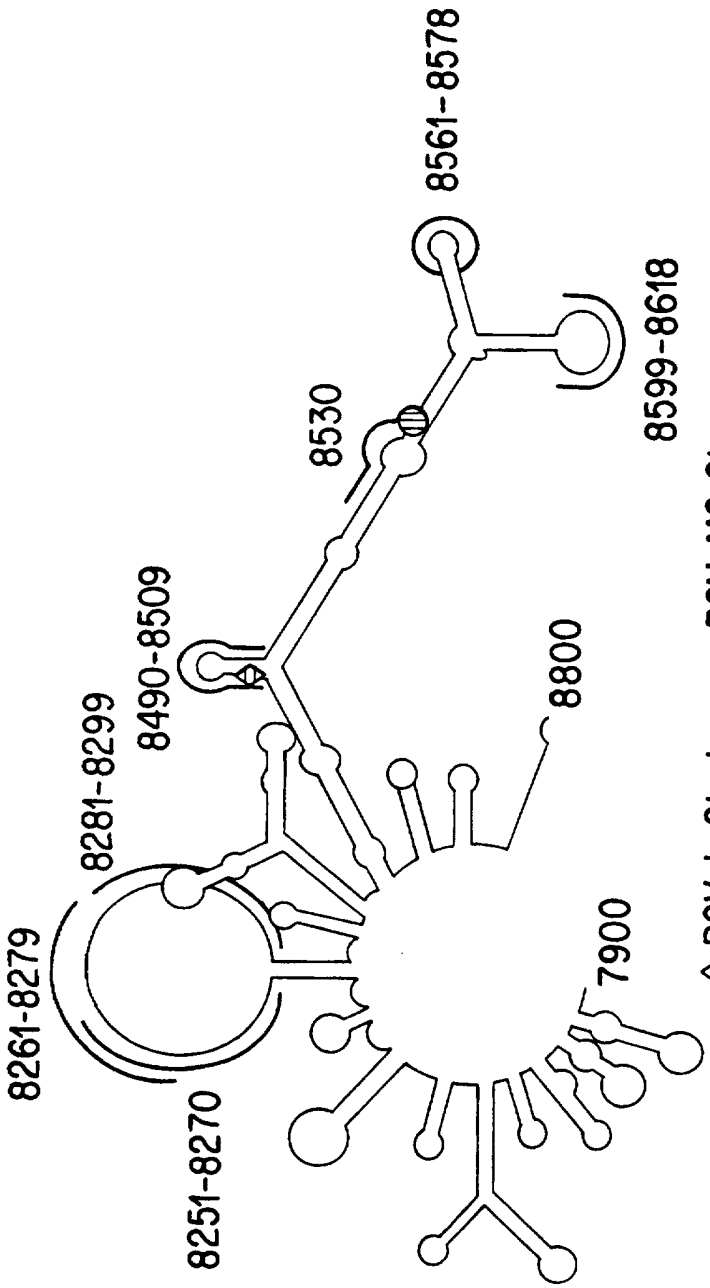
FIG.1H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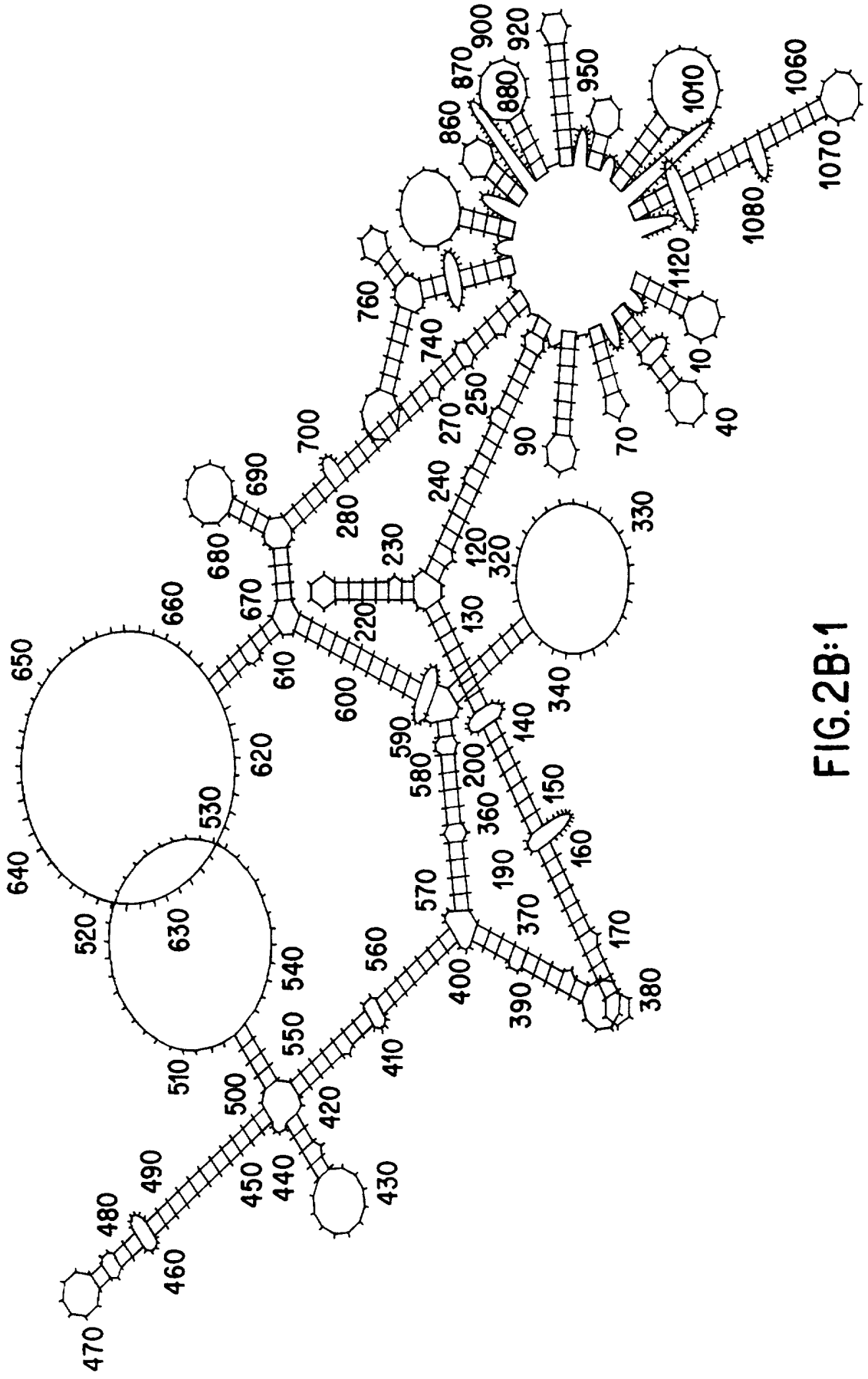
8530
8599-8618
8490-8509
8281-8299
8800
8261-8279
7900
8251-8270
RSV L Start
RSV M2 Stop
M2 Transcript: 7595-8556
M2 ORF1: 7605-8187
M2 ORF2: 8158-8421
L Transcript: 8489-15066
L ORF: 8497-14994

FIG. 2A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70
700
690
240
330
280
230
120
320
680
670
660
220
130
650
610
600
340
590
140
620
580
200
150
530
360
640
190
160
570
520
630
370
170
560
400
390
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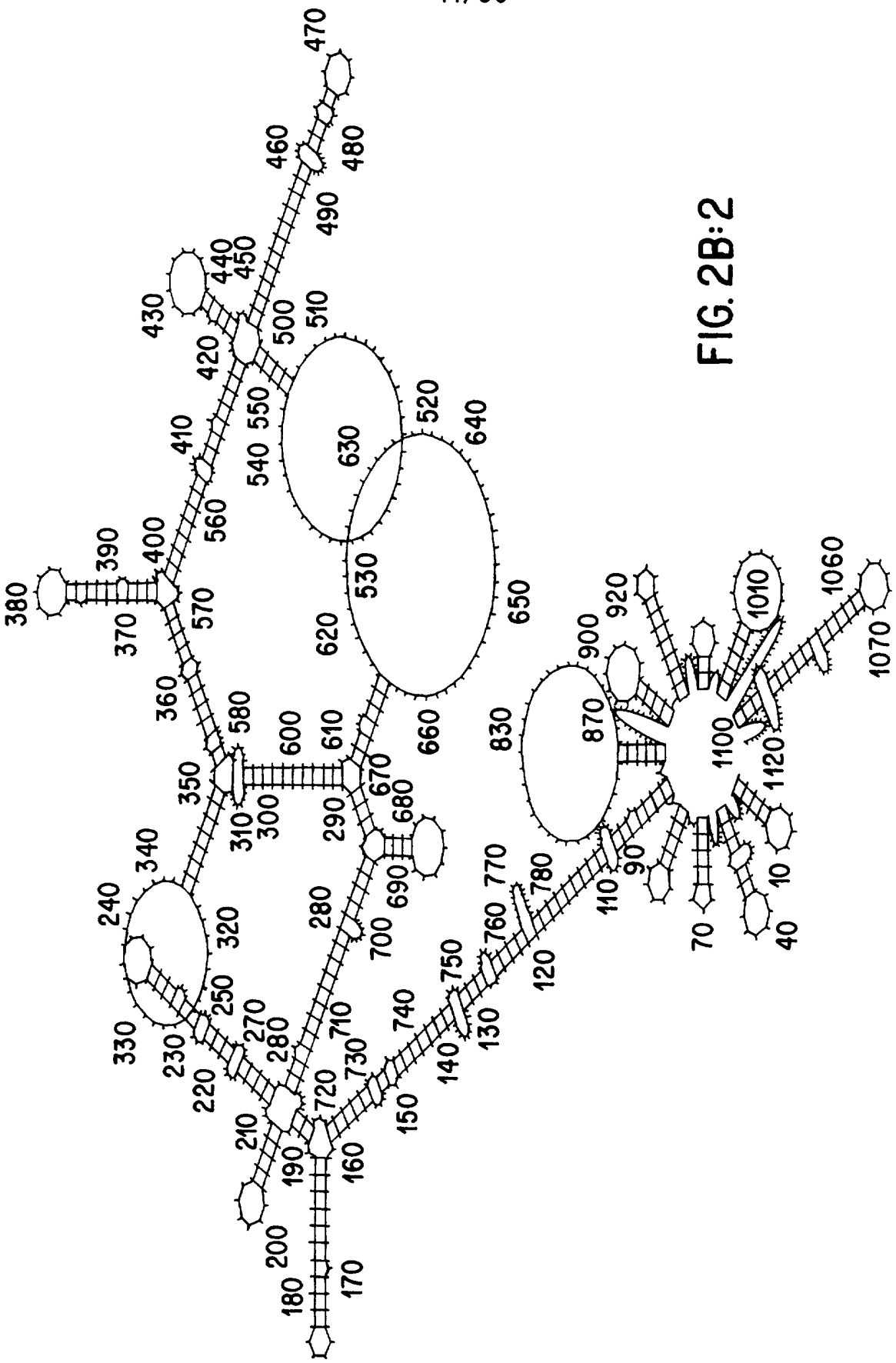
540
550
410
510
500
420
490
450
440
430
480
460
470

FIG.2B:1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380
570
370
620
650
900
920
1010
1060
1070
360
580
600
610
660
830
870
1100
1120
350
670
680
310
300
290
340
240
320
280
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90
110
10
70
40
250
270
710
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730
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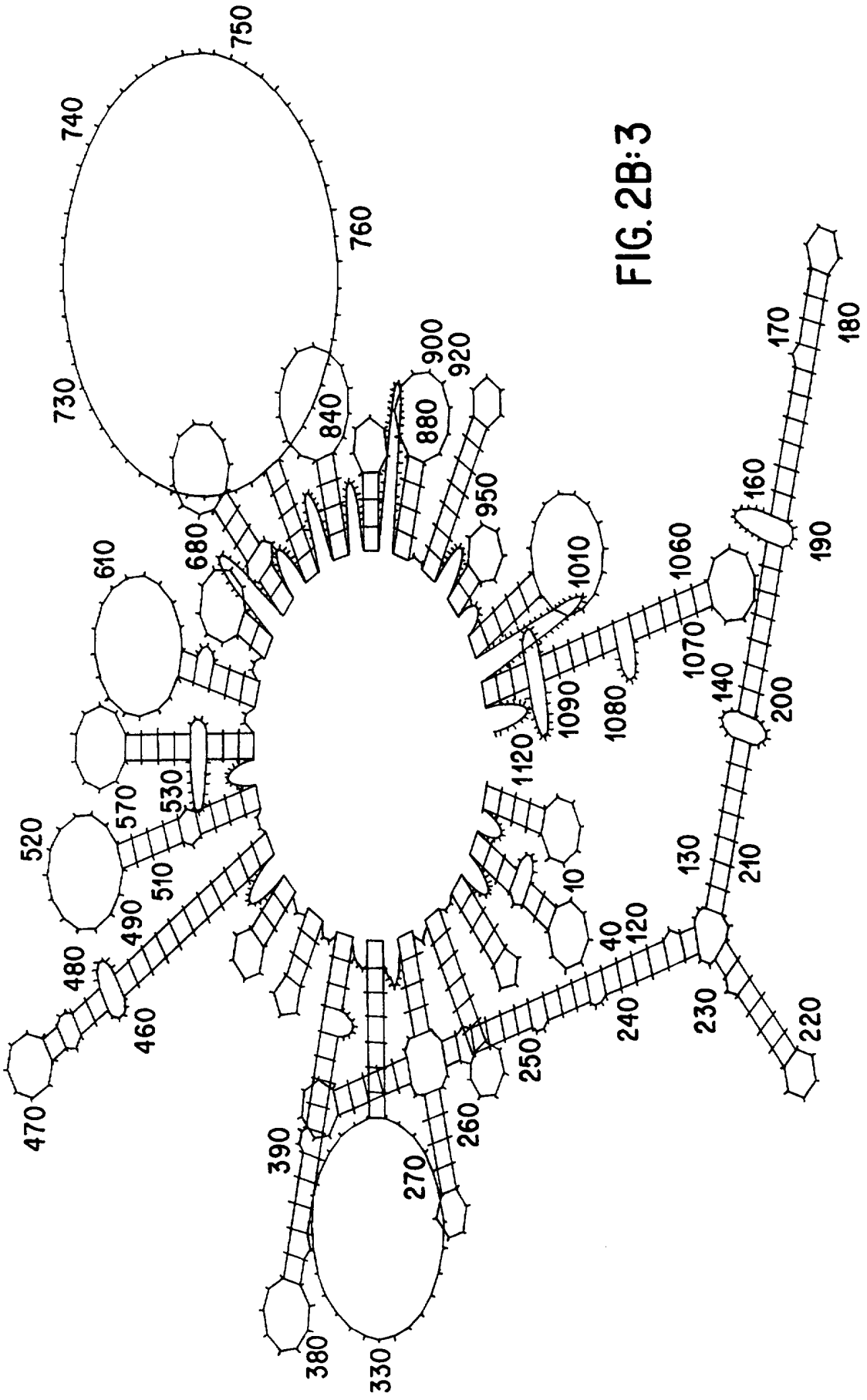
150
210
190
160
200
180
170

FIG. 2B:2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920
950
680
610
1010
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160
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180
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200
1080
1090
1120
530
570
520
510
490
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460
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10
40
120
130
210
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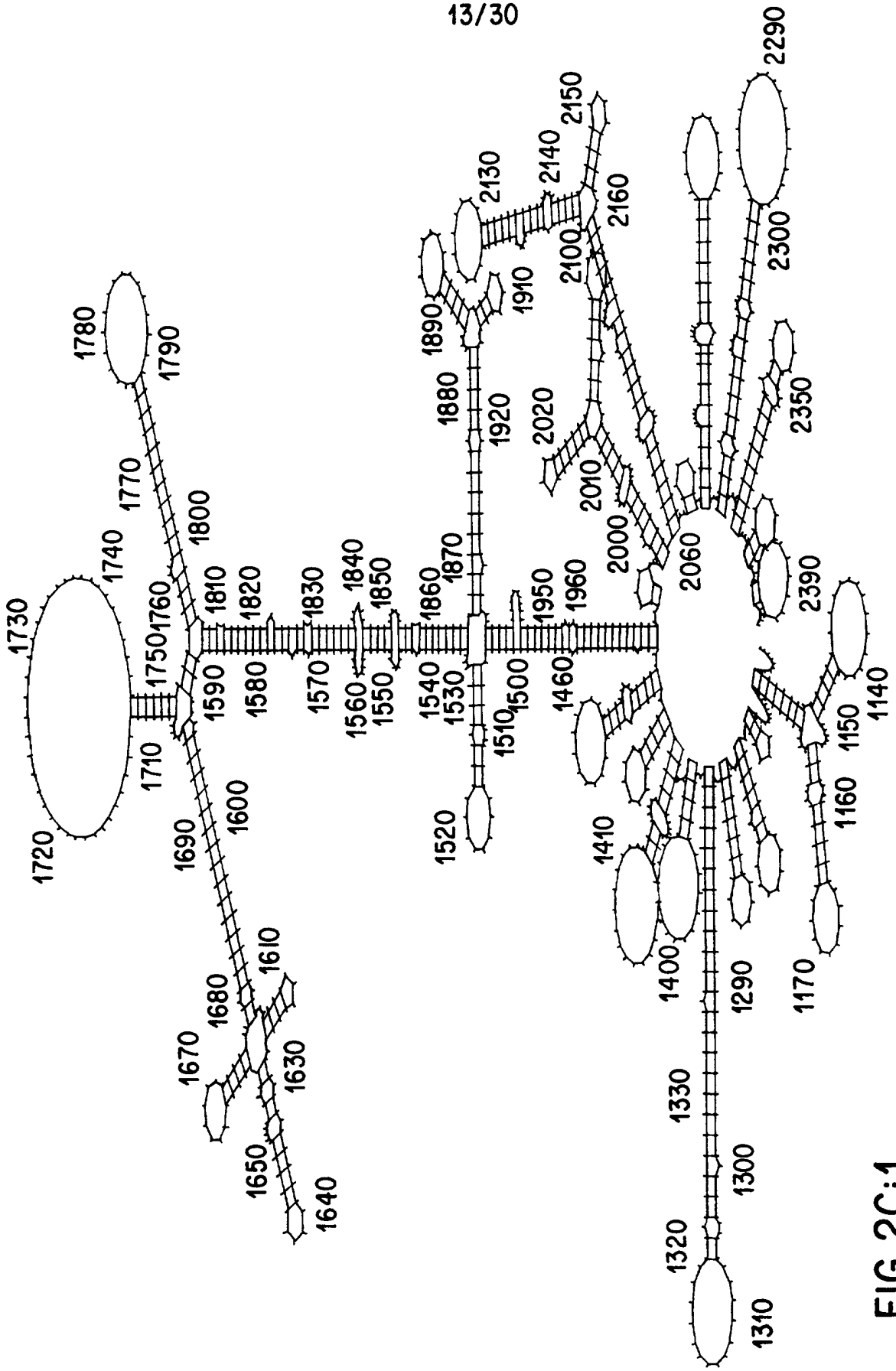
250
260
270
390
380
330

FIG. 2B:3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2020
2010
2000
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2390
1950
1960
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1710
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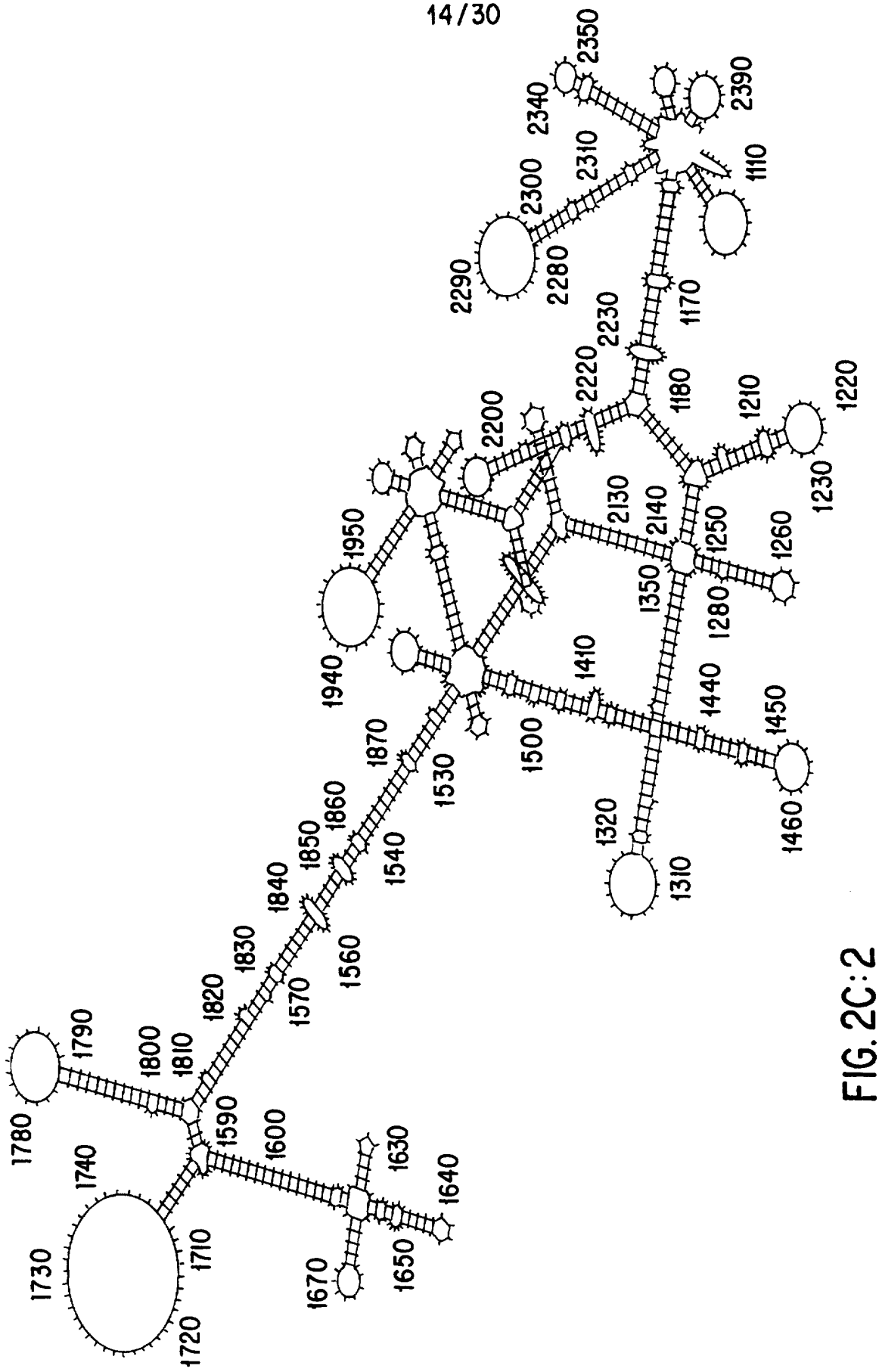
FIG. 2C:1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2140
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1950
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1560
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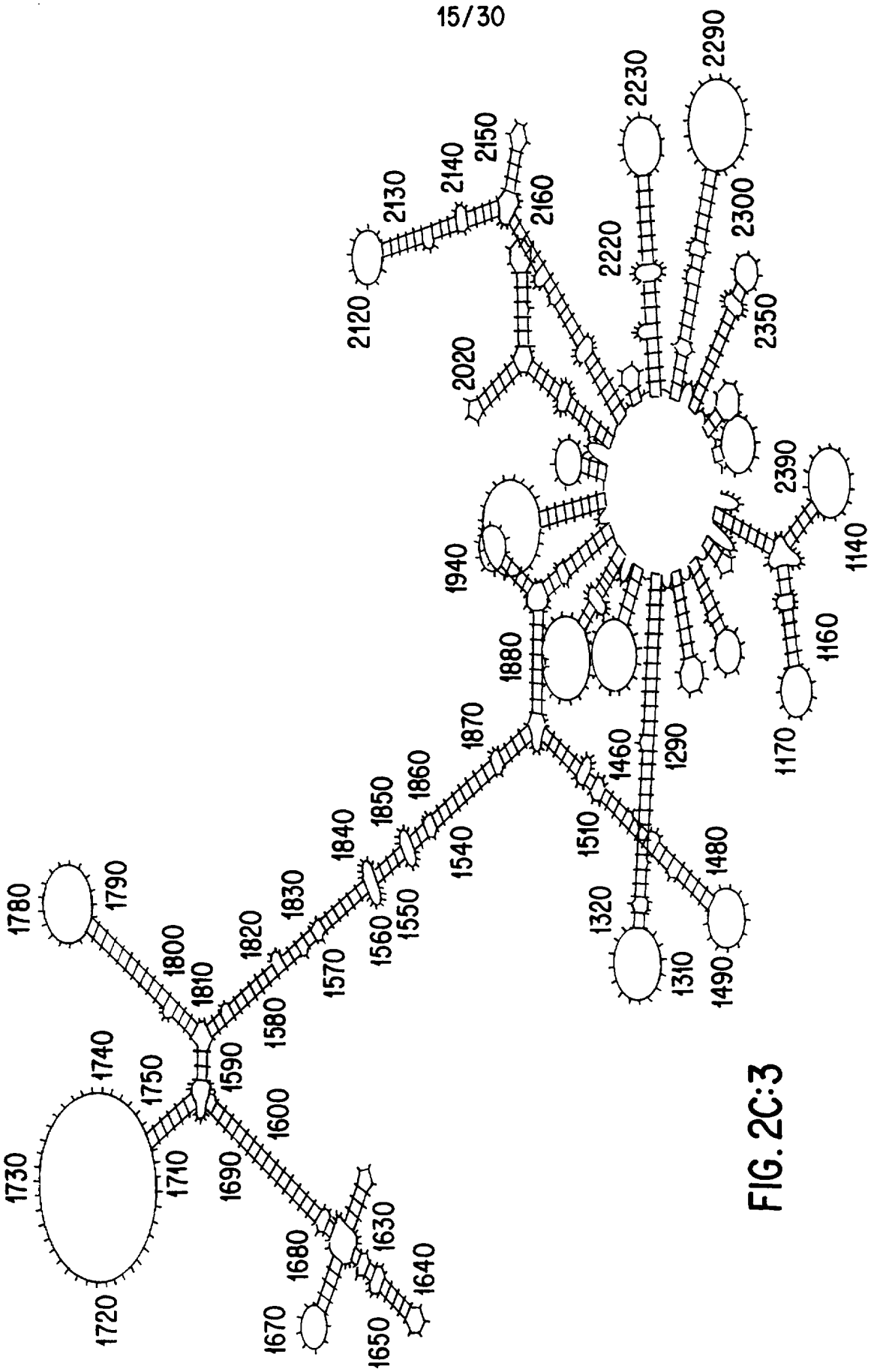
FIG. 2C:2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1570
1310
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1800
1810
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1670
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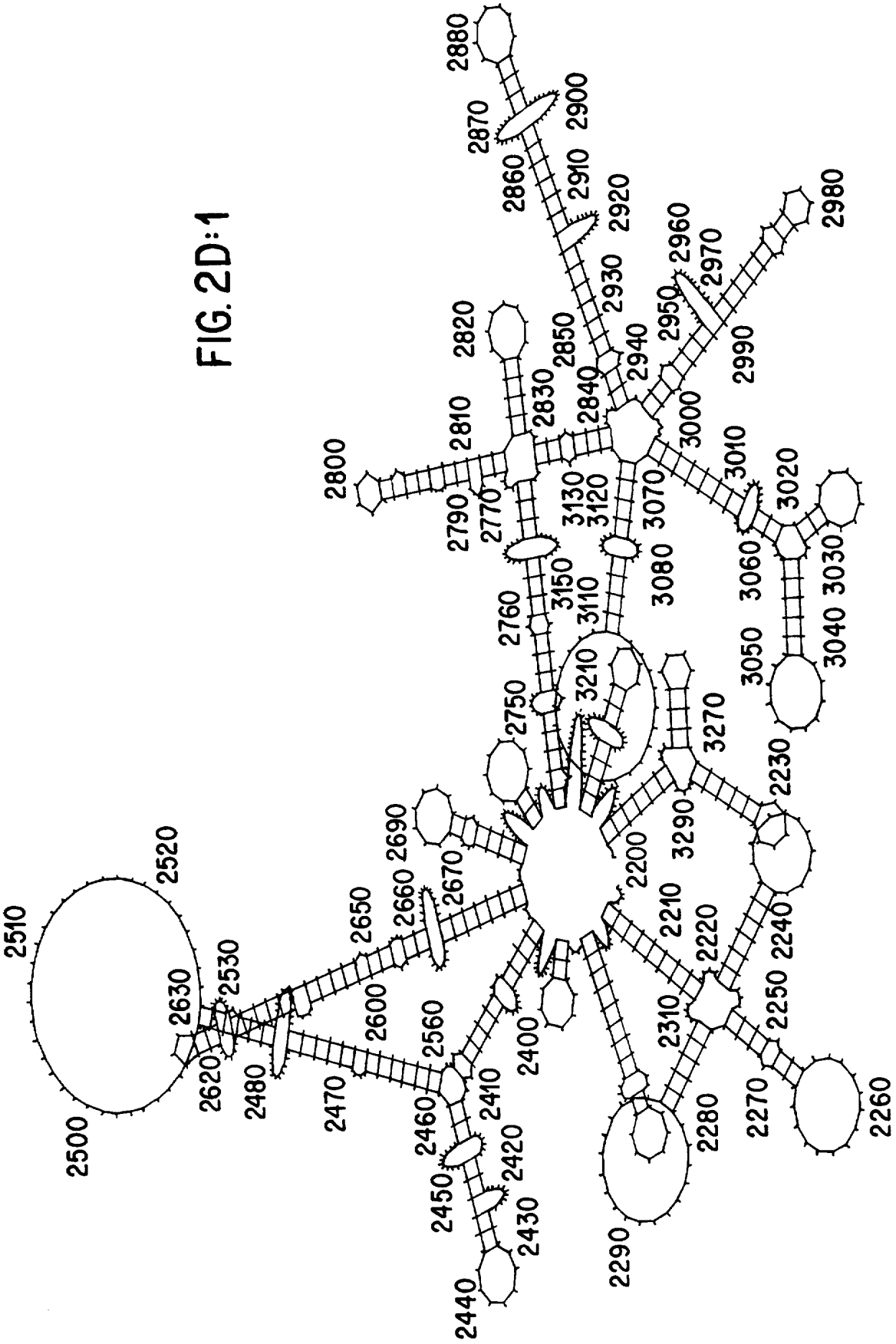
FIG. 2C:3

FIG. 2D:1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2760
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3050
2750
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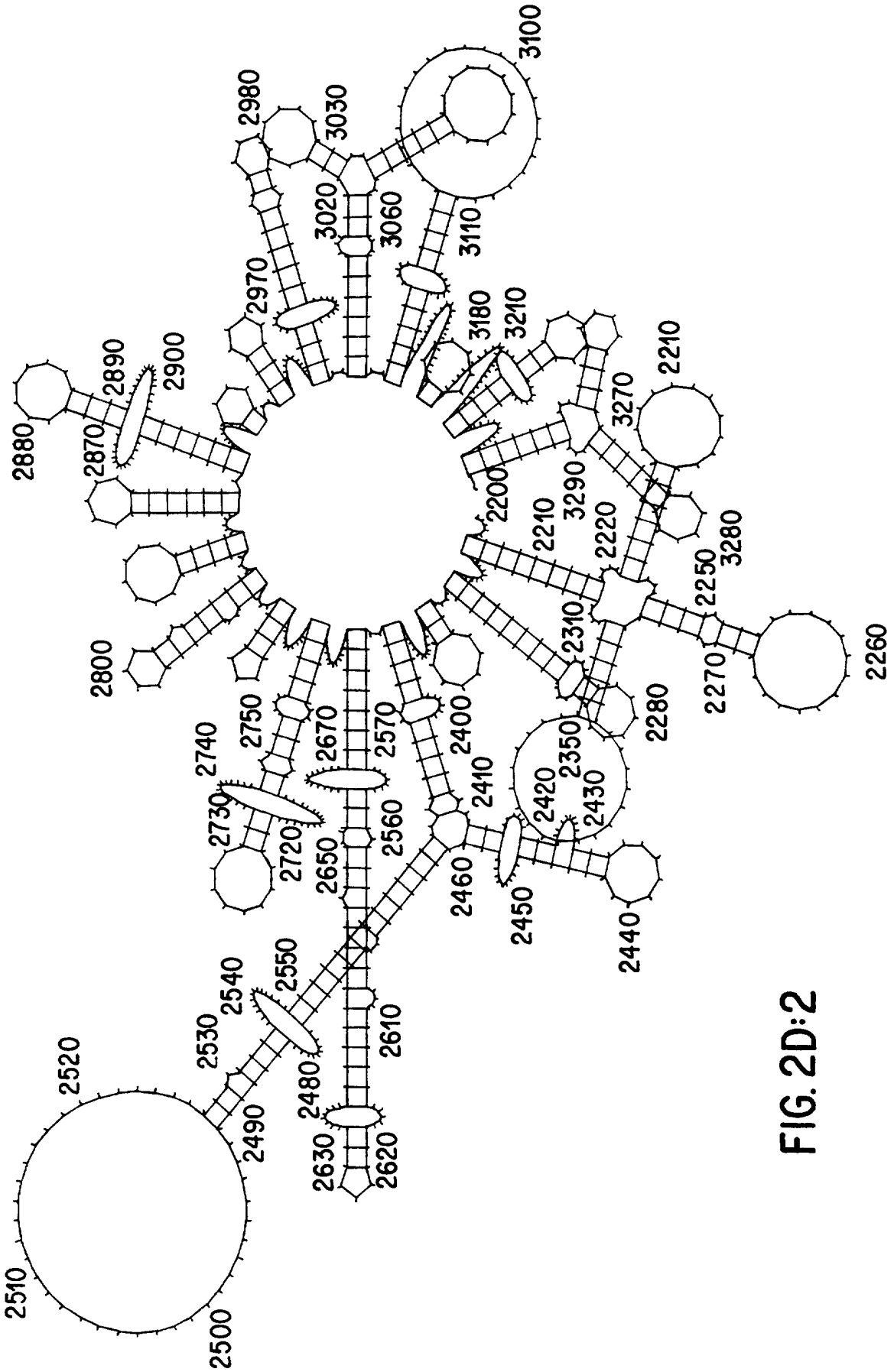
2440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2800
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2500

FIG. 2D:2

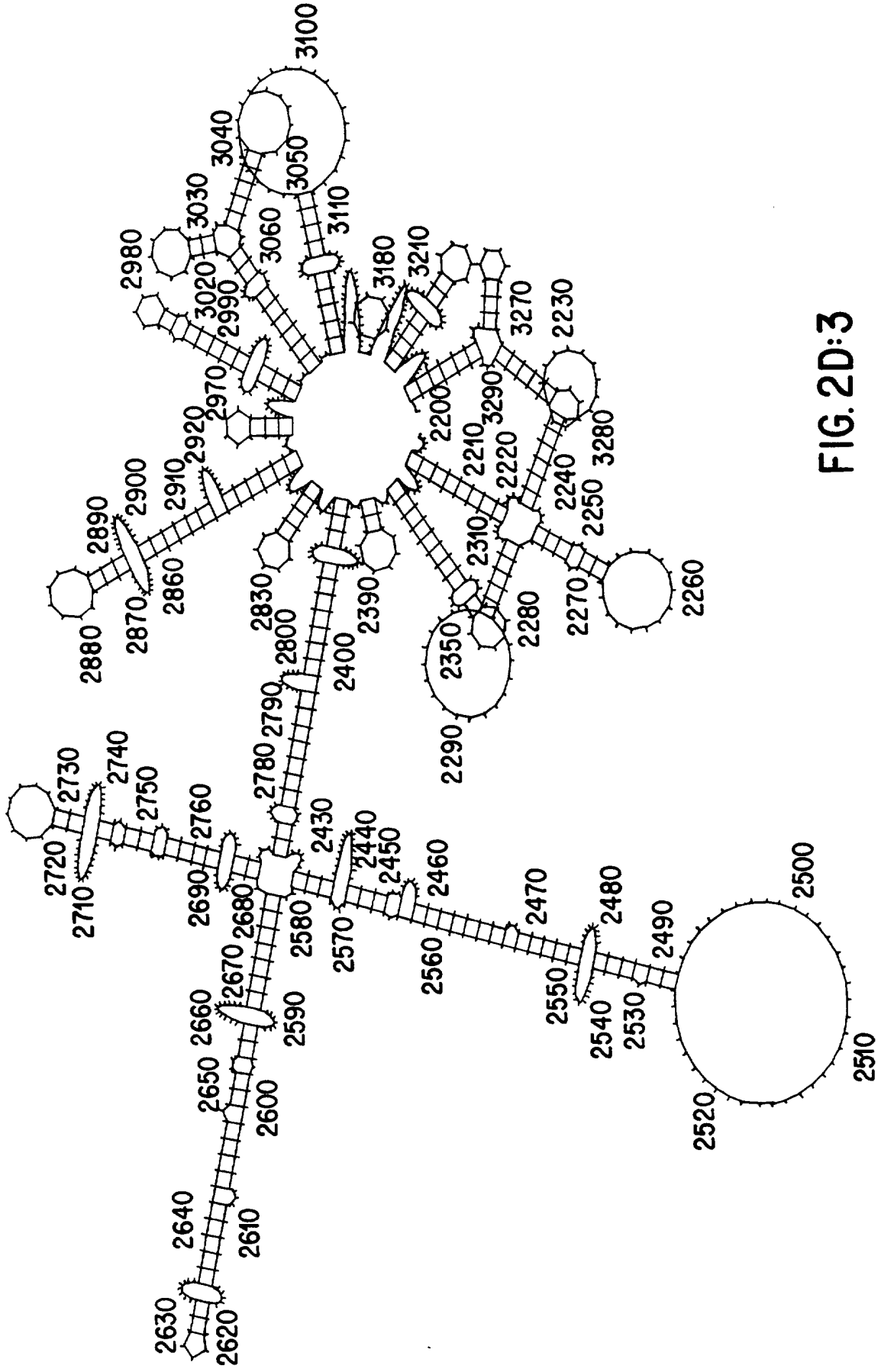
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FIG. 2D:3

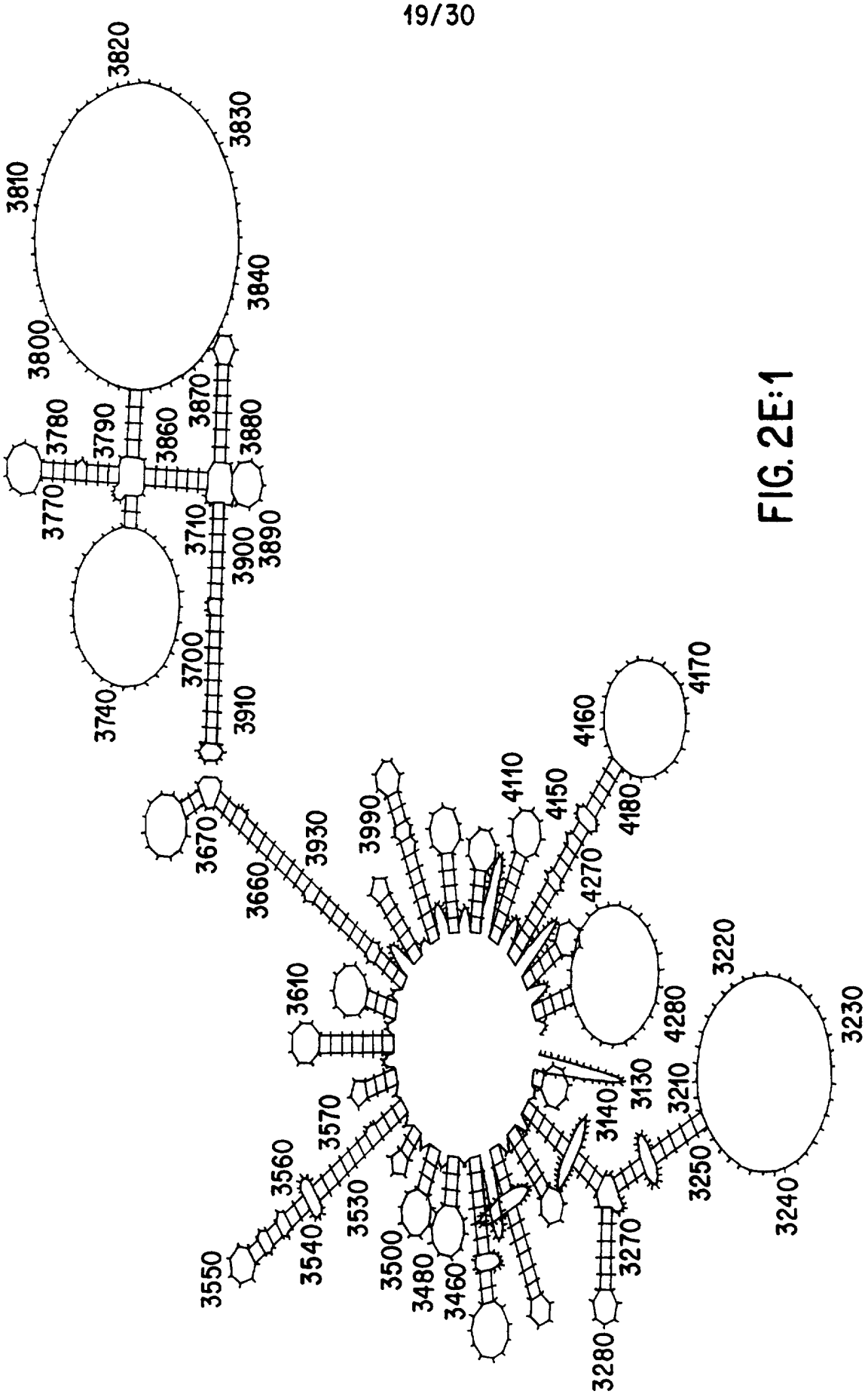
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FIG. 2E:1

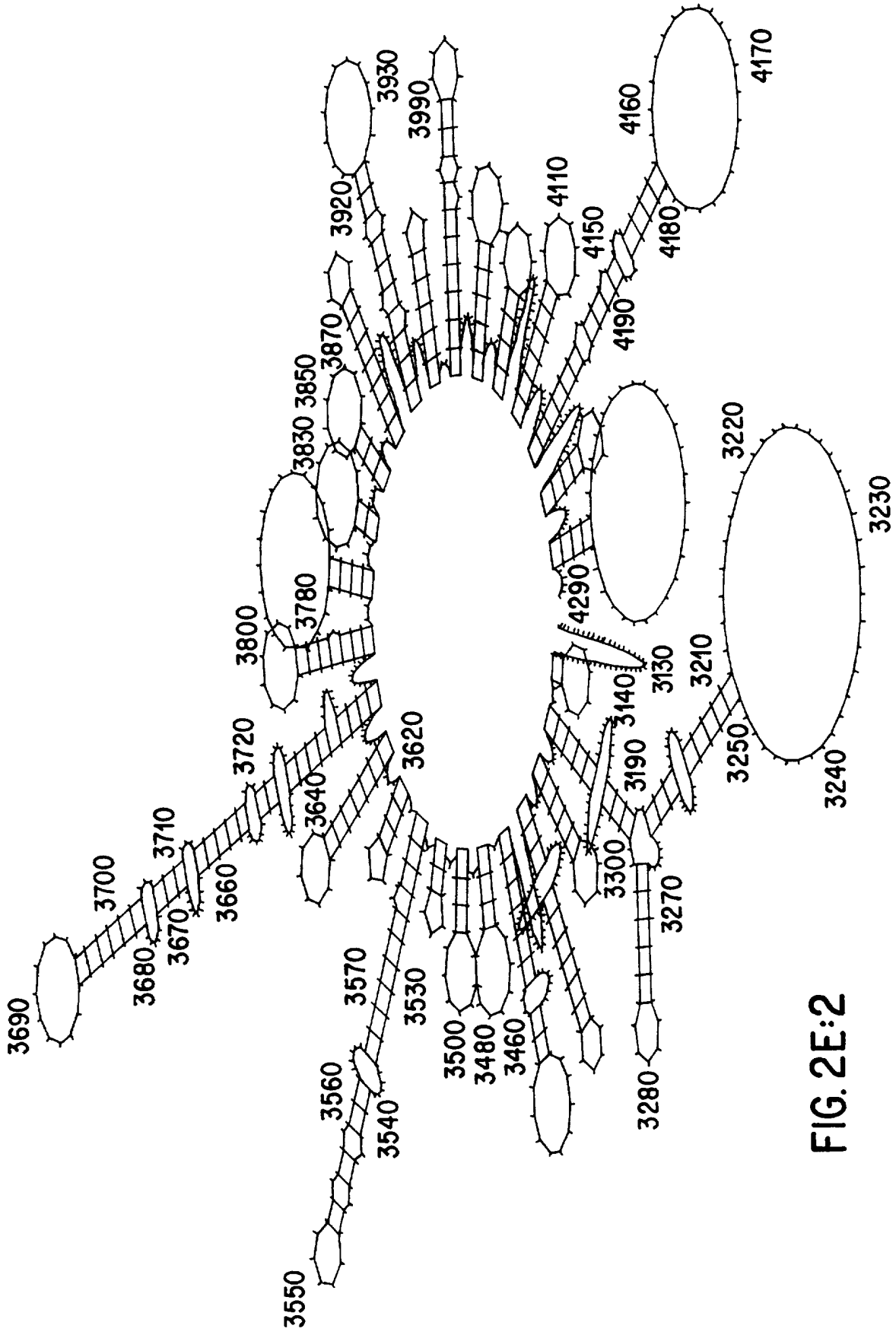
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FIG. 2E:2

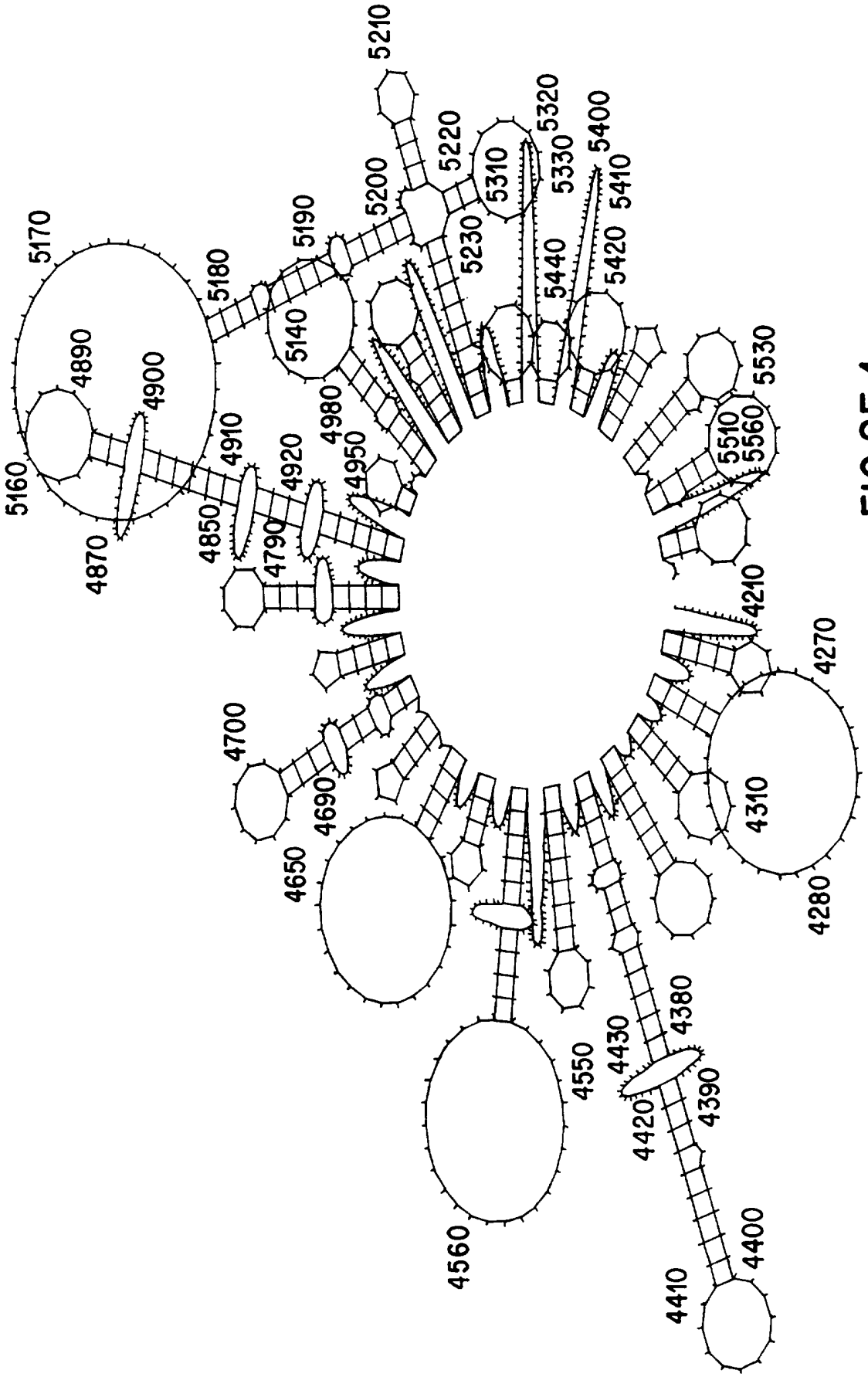
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FIG.2F:1

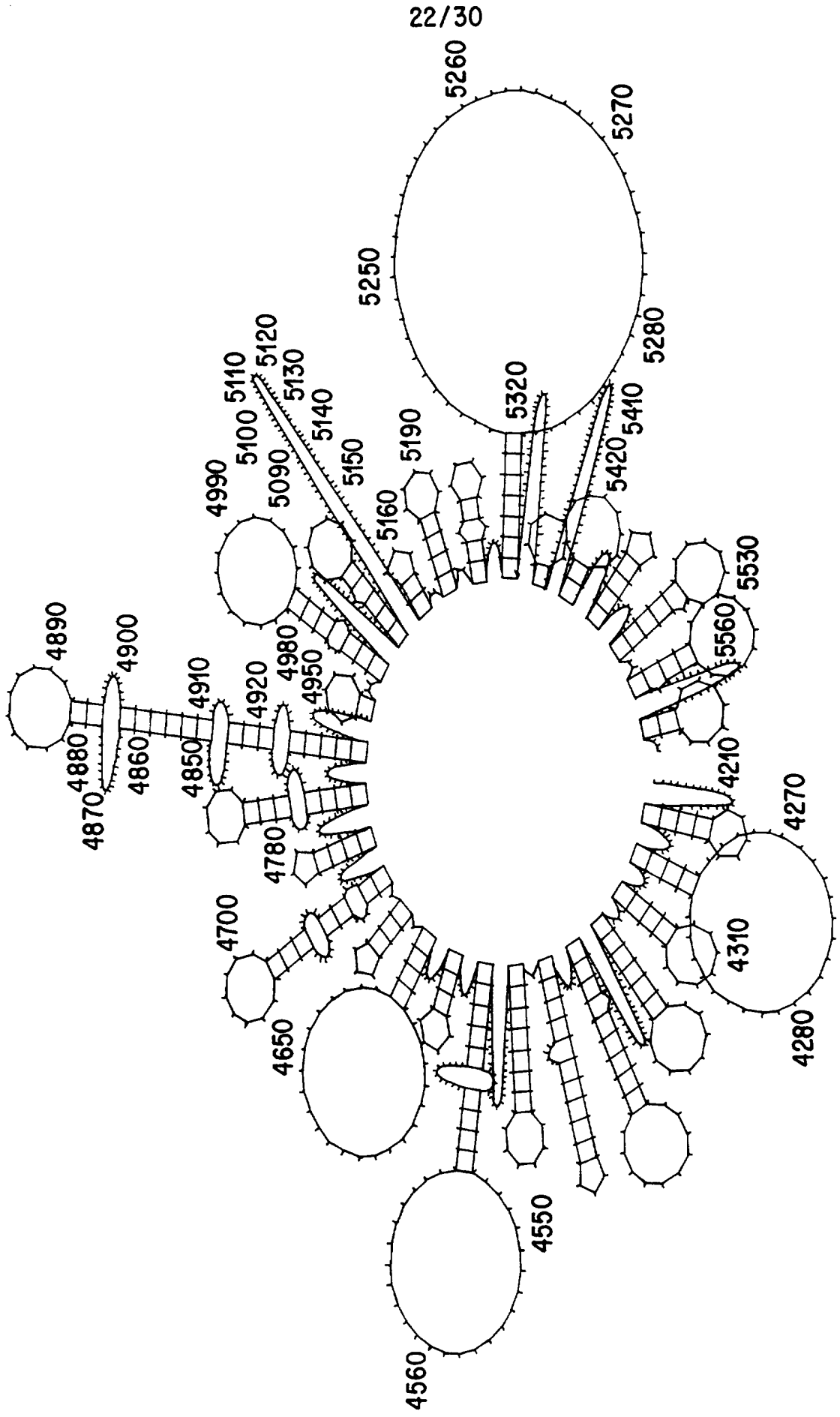
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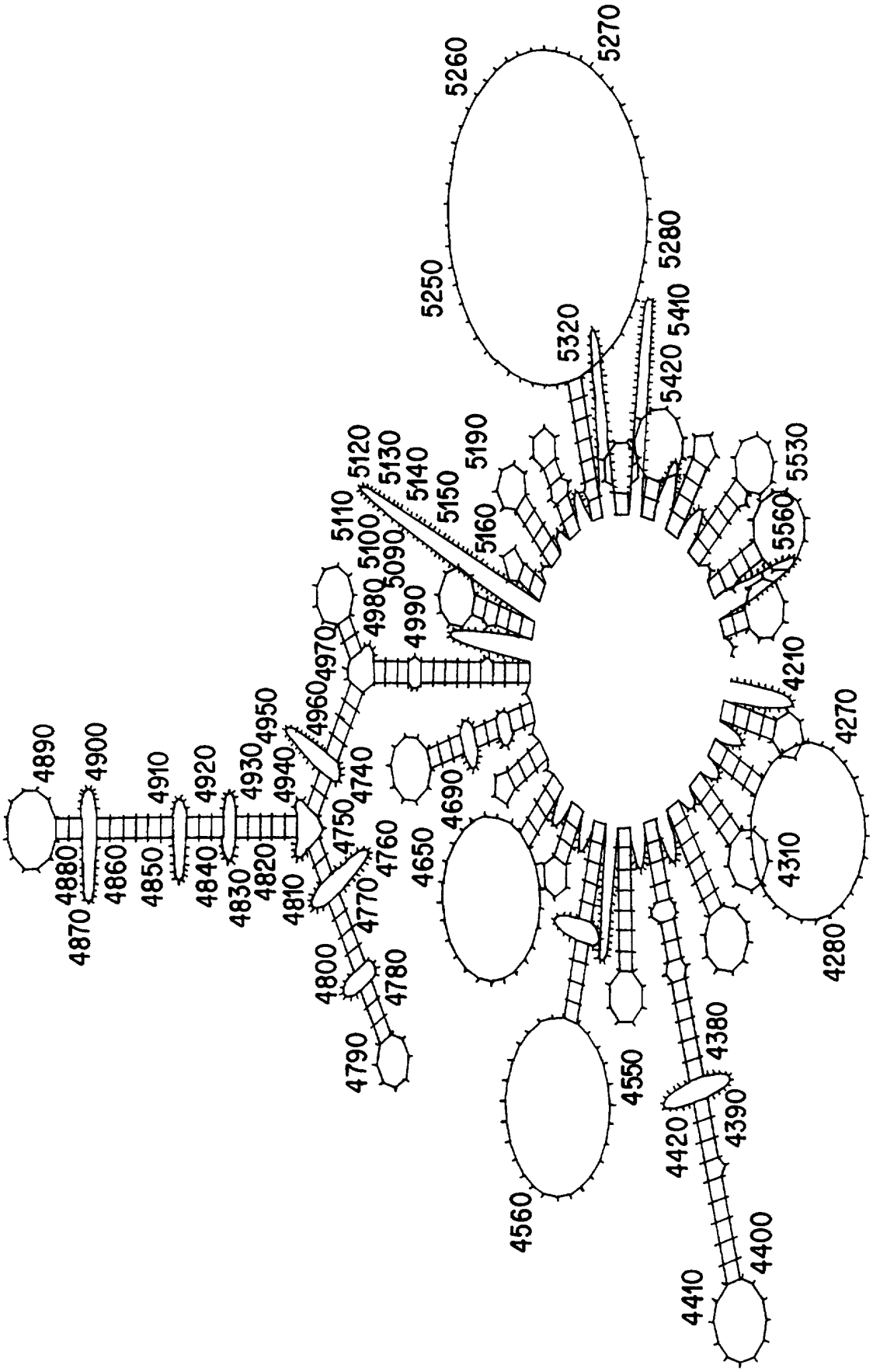
FIG.2F:2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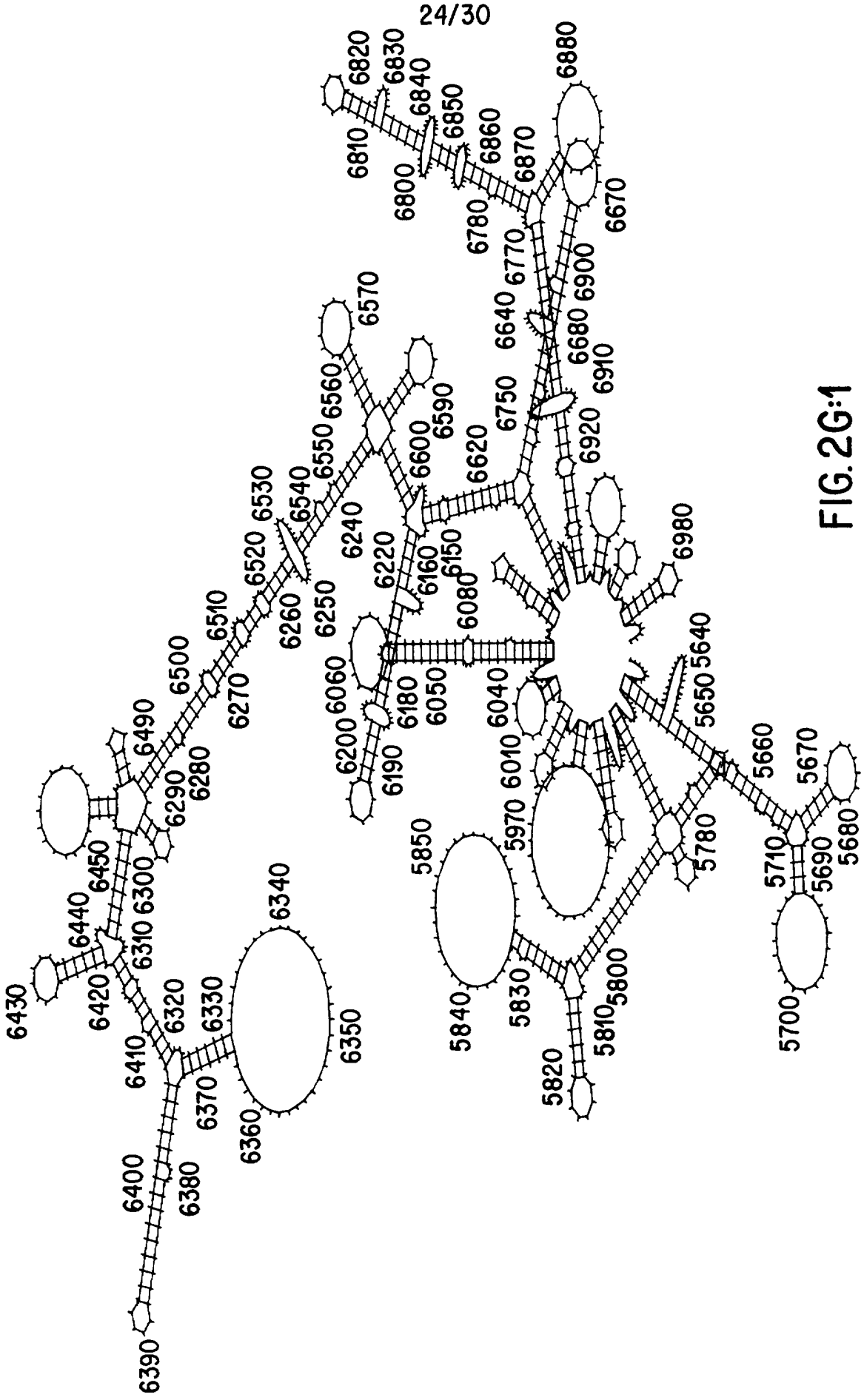
4380
4550
4420
4390
4560
4400
4410

FIG. 2F:3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6830
6840
6850
6860
6870
6880
6810
6800
6780
6770
6670
6900
6640
6680
6910
6570
6560
6590
6750
6920
6550
6600
6620
6530
6540
6240
6220
6160
6150
6980
6520
6250
6260
6080
6510
6500
6060
6180
6050
6040
5640
5650
6270
6490
6200
6190
6010
5660
5670
6280
6290
5970
5780
5850
5710
5690
5680
6450
6300
6340
6440
6310
6430
6420
6320
6330
6350
6410
6370
6360
6400
6380
6390
5840
5830
5800
5810
5820
5700

FIG. 2G:1

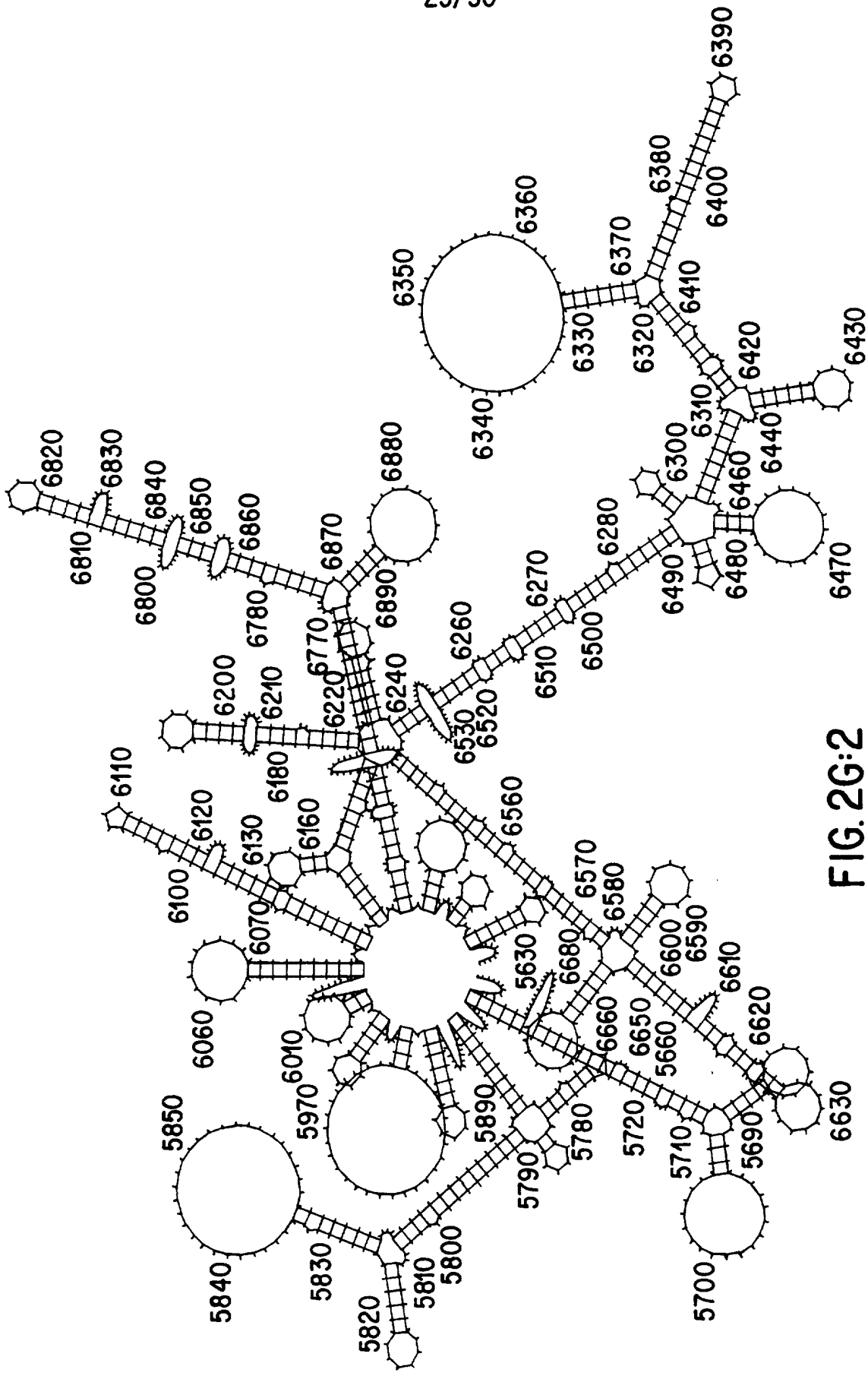
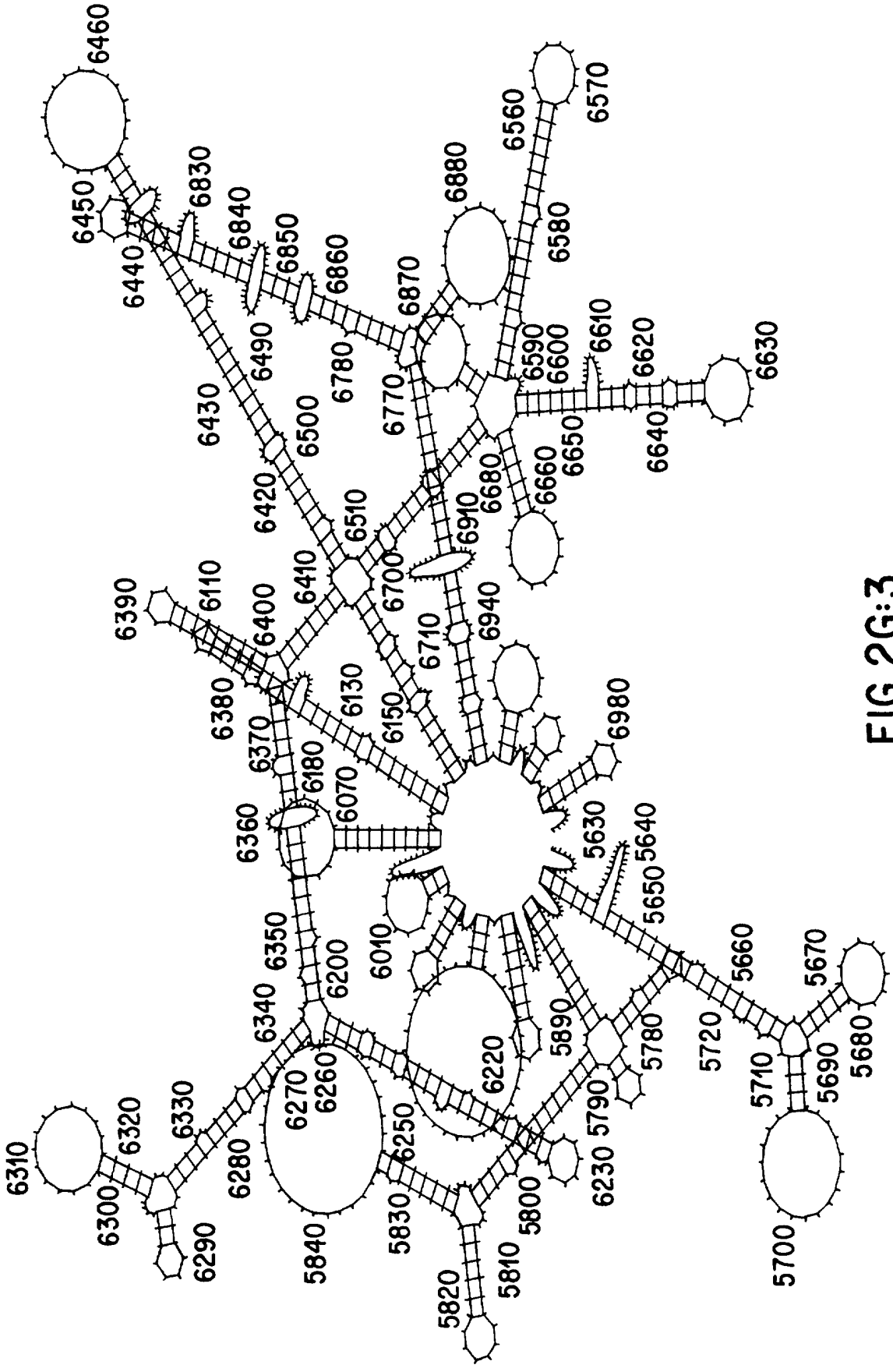

6390
6380
6400
6360
6350
6370
6410
6340
6330
6320
6420
6430
6310
6440
6300
6460
6820
6830
6840
6850
6860
6880
6870
6810
6800
6890
6780
6770
6280
6270
6260
6490
6480
6470
6500
6200
6210
6220
6240
6510
6530
6520
6110
6180
6560
6120
6130
6160
6570
6580
6100
6070
6590
6600
6680
6630
6610
6060
6010
6650
6660
6620
5970
5890
5780
5720
5710
5690
5850
5790
5700
5840
5830
5810
5800
5820

FIG. 2G:2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6850
6860
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6880
6560
6570
6580
6590
6600
6610
6620
6630
6640
6650
6660
6680
6910
6490
6780
6770
6500
6430
6420
6510
6700
6410
6400
6110
6390
6710
6940
6130
6150
6980
6380
6370
6180
6070
6360
5630
5640
5650
6350
6010
6200
6340
6220
5890
5780
5720
5660
5670
5680
5690
5710
6330
6320
6310
6270
6260
6250
6230
5790
6280
6300
6290
5840
5830
5800
5810
5820
5700

FIG. 2G:3

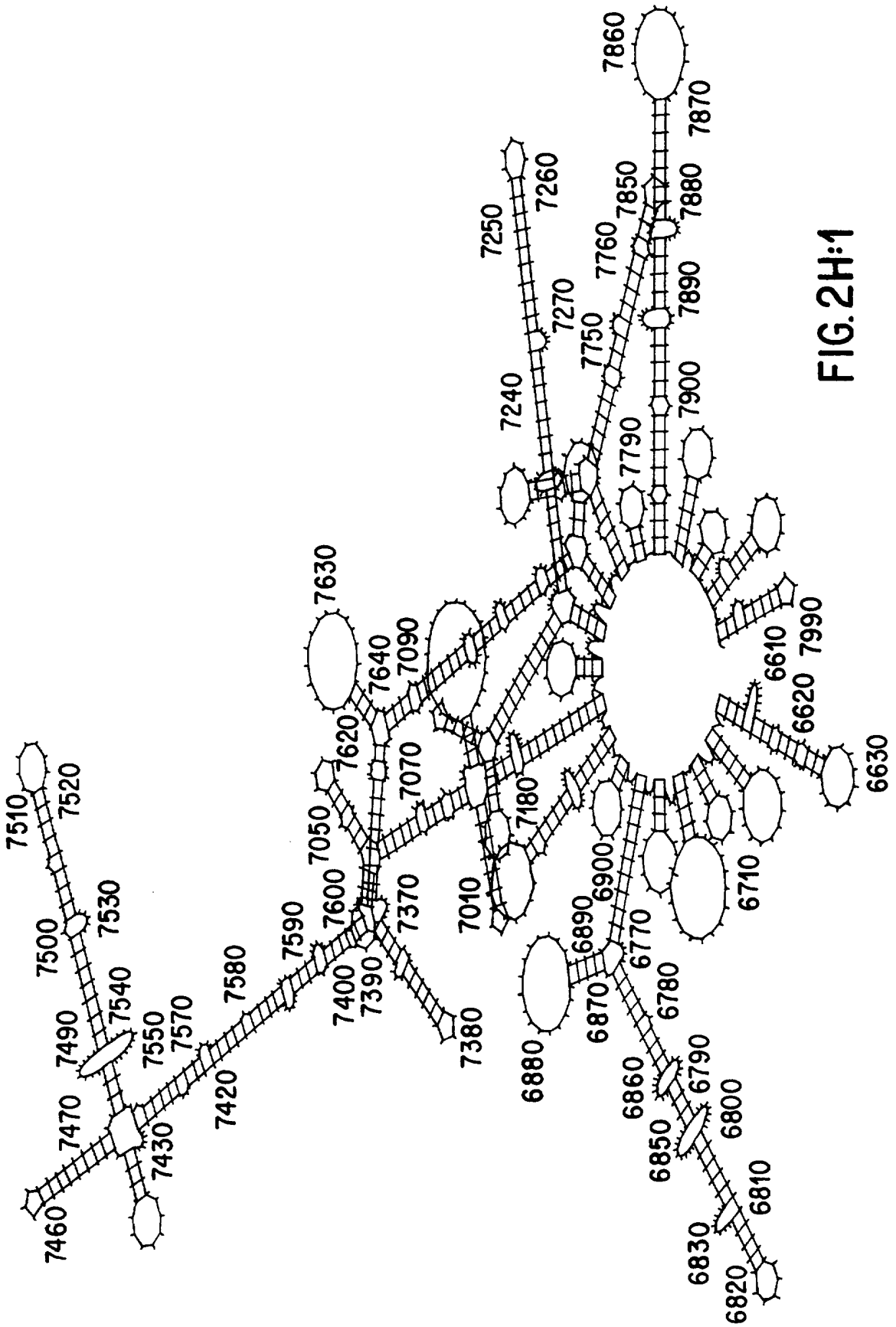
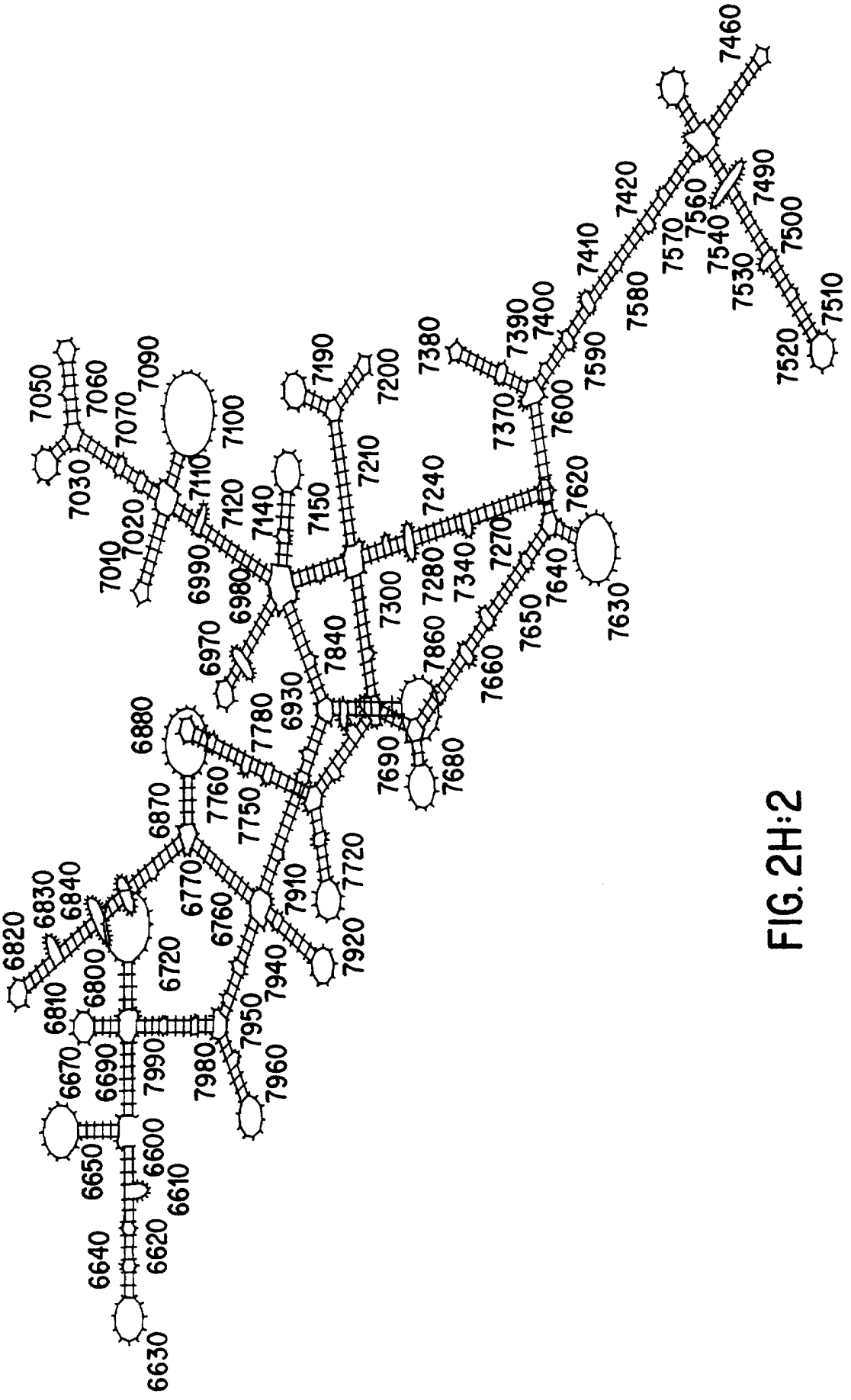

7860
7870
7250
7260
7850
7880
7760
7890
7270
7750
7240
7900
7790
7630
7640
7090
6610
7990
6620
7620
7070
6630
7180
7510
7520
7050
6900
7600
7370
7010
6710
7500
7530
6890
6770
7590
7400
7390
6780
7540
7580
6870
7490
7550
7570
7380
6880
6860
6790
7420
6850
6800
7470
7430
6810
7460
6830
6820

FIG.2H:1


6630
6640
6620
6610
6600
6650
6670
6690
6990
6810
6800
6820
6830
6840
6720
6770
6760
6870
6880
6970
6980
6930
7780
7760
7750
7840
7300
7690
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7630
7340
7280
7270
7240
7620
7600
7590
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7140
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7110
7100
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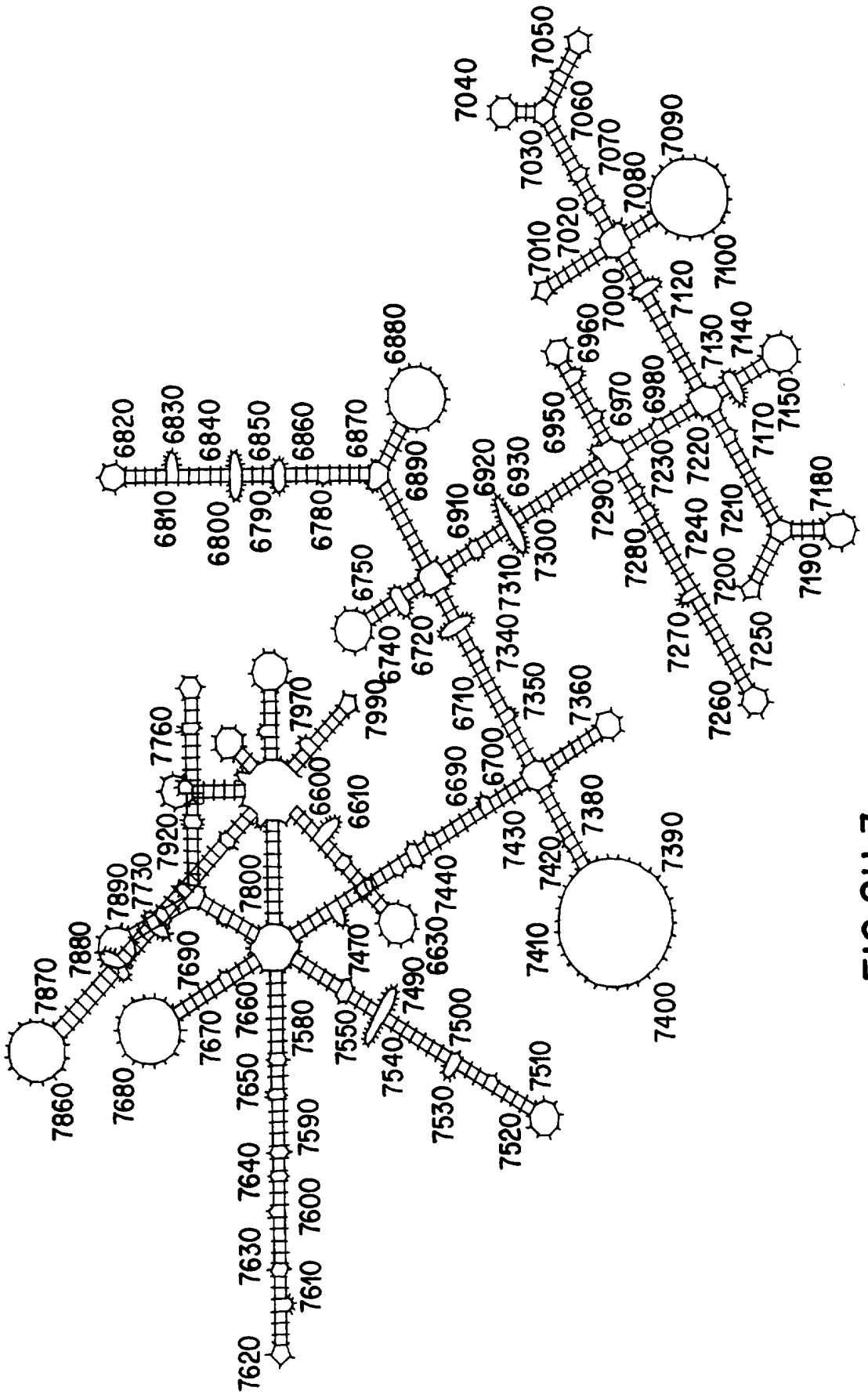
7950
7960
7980
7990

FIG. 2H:2


7040
7050
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7090
7080
7010
7020
7000
7100
7120
6960
7130
7140
6880
6820
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6980
7150
7170
6920
6930
6910
7230
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7180
6810
6800
6790
6780
7290
7300
7240
7210
7190
6750
7310
7280
7200
6740
6720
7340
7270
7250
7260
7990
7970
6710
7350
7360
7760
6600
6700
6690
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7920
7380
7430
7420
7390
7890
7730
7800
7440
7410
6630
7880
7470
7400
7870
7690
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7610
7620

FIG. 2H:3

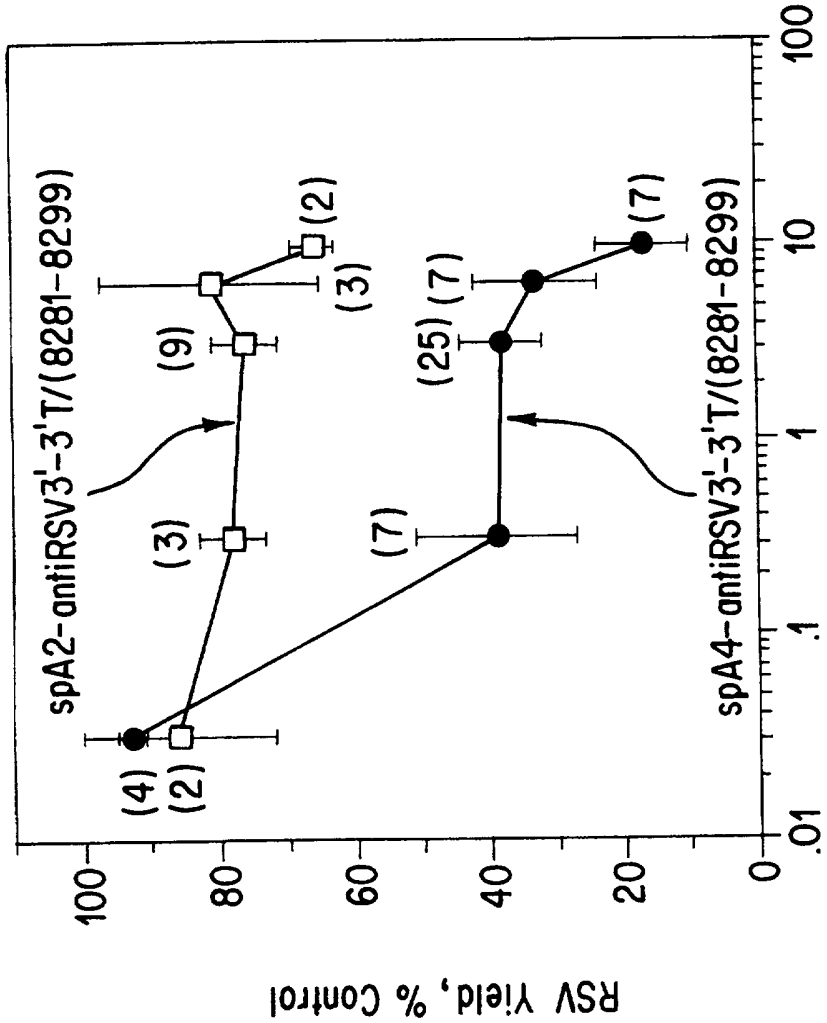
RSV Yield, % Control
100
80
60
40
20
0
.01
.1
1
10
100
Oligonucleotide, uM
spA2-antiRSV3'-3'T/(8281-8299)
spA4-antiRSV3'-3'T/(8281-8299)
(4)
(2)
(3)
(9)
(2)
(3)
(7)
(25)
(7)
(7)

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02531

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70; C07H 21/00, 21/02, 21/04
US CL : 514/44; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : US: 435/ 199, 375; 514/44; 536/23.1, 24.5
IPC: A61K 31/70; C07H 21/00, 21/02, 21/04; C12N 5/08, 9/22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN (BIOSIS, MEDLINE, CAJACS, CAPLUS, INPADOC, USPATFULL, WPIDS).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PANUSKA et al. Respiratory Syncycial Virus Induces Interleukin-10 by Human Alveolar Macrophages. Journal of Clinical Investigation. November 1995. Vol. 96, pages 2445-2453, especially abstract, page 2445, column 2, to page column 1, and page 2451, column 1, through page 2452, column 1.	1-7, 12-17
T	CIRINO et al. Targeting RNA Decay with 2', 5', oligoadenylate-Antisense in Respiratory Syncycial Virus-Infected Cells. Proceedings of the National Academy of Sciences, USA. March 1997, Vol. 94, pages 1937-1942, especially abstract, page 1937, column 1 through column 2, and page 2941, column 2, through page 2942, paragraph bridging columns 1 and 2.	1-7, 12-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 JUNE 1997	Date of mailing of the international search report 02 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THOMAS G. LARSON, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02531

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,532,130 A (R. ALUL) 02 July 1996, especially abstract and column 6, line 45 to column 12, line 28.	1-7, 12-17
Y	MAITRA et al. Catalytic Cleavage of an RNA Target by 2-5A Antisense and RNase L. The Journal of Biological Chemistry. 23 June 1995, Vol. 270, No. 25, pages 15071-15075, see entire document.	1-7, 12-17
Y	WO 95/22553 A1 (HYBRIDON, INC.) 24 August 1995, see entire documnet.	1-7, 12-17
Y	WO 94/09129 A2 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES) 28 April 1994, see entire document.	1-7, 12-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02531

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 8-11 and 18-19
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 8-11, and 18-19 recite limitations involving specific nucleic acid sequences. However, the required sequence identifiers (SEQ. ID. NOS.) were not provided for these sequences. Therefore, it was not possible to execute a sequence data base search for the sequences recited in these claims.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.