Attachments:

A: January 7, 1988- Letters detailing the start of the collaboration between Robert and Phil Felgner. First invention disclosure concerning idea of use of mRNA as a drug.

B: March 7, 1989- Disclosure from Vical elaborating of findings and ideas generated by Robert, Phil and John as of that date. Notice the absence of information concerning "Naked" polynucleotides, any discussion of use of "free" polynucleotides etc. At this time, in vivo experiments had been done, but no in vivo "negative controls" of RNA without added lipid had been performed.

C: March 7, 1989 and March 14 1989. First record of an in vivo experiment involving the negative control of CAT mRNA without added lipid. Data showing weak positive result with mRNA lacking lipid. Note that the experiment was planned by Robert, and sent as blinded samples to John Wolff. Negative control was discussed with me and another Vical employee (name available upon request) prior to Dr. Felgner and Dr. Wolf's involvement with the experiment.

D: June 14, 1989- First experiment involving "Naked DNA" injection. Notice again that Robert had continued to design the experiments and provide blinded samples to Dr. Wolff.

E: June 16, 1989- Internal Vical document prepared by Phil Felgner summarizing current status and plans for Vical gene therapy program. At that time, the Vical program had one employee dedicated to gene therapy research- Robert.

F: June 1, 1989- Invention Disclosure showing Dr. Felgner signing off on documents (in this case a disclosure on the use of RNA) when he didn't contribute intellectually. Instead of signing off as witness, he wrote his own name above Robert's-despite there obviously being no room, as Robert had purposefully written and signed the disclosure himself, as it had been his idea, not Dr. Felgner's. This pattern was repeated again and again by Dr. Felgner.

G: August 1, 1989- Roberts resignation letter from Vical.

Appendix A

THE SALK INSTITUTE

1/7/88

Dr. Phil Felgner
Syntex Research
3401 Hillview Avenue, P.O. Box 10850
Paolo Alto, Ca. 94303

Dear Phil,

Thanks for your patience in waiting for these clones for your DOTMA RNA transfer research. I hope my heroic procrastination has not inconvienienced you too much.

Enclosed are the plasmids pSP64T, pSPCAT (from Peter Walter's lab), pSP64A (a Promega plasmid) and p31T (a construction of mine). Also included are maps and sequence information on these plasmids, and the Krieg/Melton paper describing pSP64T. pSP64T has the Xenopus beta-globin 5' untranslated region and polyA tail under the control of the SP6 promoter. p31T is similar, being derived from the phagmid IBI31 and having the pSP64T b-g insert placed under the control of the T7 promoter (I prefer T7 for longer inserts). pSPCAT is pSP64T with CAT coding sequences placed within the b-g context under the control of SP6. pSP64A is useful if you wish to place a CDNA with it's own 5' untranslated region under the control of SP6 and generate a polyA tail while transcribing in vitro.

I am also sending you a copy of a T7 capping protocol which I know works well. However, my bias is that your lack of positive results from mRNA transfer attempts with your lipid is most likely due to one of two factors: first, that including a poly A tail will greatly increase the stability of your transfered message, and second that I have been sucessful demonstrating transfer with a reporter gene which is claimed by Suresh Subramani to be 10^2 to 10^3 fold more sensitive than CAT. The plasmids which I am sending should eliminate the first problem, and I am still hassling with the lawyers to get you the Luciferase clones so that the second problem might be resolved.

I am also sending a XEROX copy of the results alluded to above. Please reguard these as very preliminary. The RNA concentrations are only estimates from Ethidium stained TBE/agarose gels, and the RNA samples had not been extensively purified and hence also included DNA template, BSA, and unincorporated NTP's (all potential inhibitors of DOTMA-RNA interactions). I am working out a new method of purification

which may eliminate these competitors (especially the Pi and NTP's).

On another level, I have had some interesting discussions with others around the lab concerning these liposomes. One issue which I find particularly intriguing is the localization of the nucleic acid relative to liposome interior or exterior. A simpleminded experiment would be to DNA'se treat liposomes after mixing with DNA and then extract and run the resulting purified nucleic acid out on a gel, looking for protection of degradation by the liposome. Another issue is the relative efficiency of supercoiled versus linear DNA transfer. Yet another is single stranded DNA transfer, as single stranded DNA is reported to be more efficient for gene conversion.

Finally, thank you for the Lipid mixtures, and for the opportunity to explore the potentials of your preparation. The simplicity and efficiency of your technique makes it very appealing to me and others in the lab, and I am very encouraged by the preliminary results concerning RNA transfer. Hopefully by the end of the month I can have the RNA transfer characterized adequately for inclusion in a publication.

I hope this information and these reagents will be useful in your work. Give me a call when you get around to it.

Best wishes,

Robert Malone

THE SALK INSTITUTE

1/21/88

Dr. Phil Felgner
Syntex Research
3401 Hillview Avenue, P.O. Box 10850
Paolo Alto, Ca. 94303

Dear Phil,

This is just a note reguarding the enclosed data (which I hope you will find encouraging). The construction which I used initially to generate the data sent to you previously was T7LUCFR. This required in-vitro post transcriptional polyadenylation of the RNA generated, and as I think I indicated to you previously, this process was a bit of a headache. I now have a new construction ($T7LUCFRA_n$) which places luciferase coding sequences devoid of a 5' untranslated region and a 3' polyadenylation signal in the context of the xenopus beta globin 5' and 3' untranslated regions and generates an A tail during the primary transcription.

RNA generated from the old construction at the same time as that used to generate the data previously sent, and used at the same concentration, was used for the time course evaluation. This involved an incubation of RNA + DOTMA for 3h in Opti-mem, after which the media was changed to DMEM + 10% FCS and the first time point taken (time 0). The cells were NIH 3T3. As I think you can see the time course needs to be pushed back to the point when the DOTMA is first added to the cells.

The second experiment (RNA concentration effects) was performed with RNA generated from the new construction, then DNA'se treated, then passed over a Sephadex G50 column. I am frankly astounded at the level of activity observed. With this technique we should be well within the sensitivity of CAT. Hopefully I will be trying out CAT next week (also co-transfection). Please note that the harvest time was sub optimal and still the activity was quite high.

Also enclosed is a summary of one of the potential applications which we discussed yesterday. I think it is an application worth pursuing further.

I hope you get the chance to come down here and say hello. Until then,

best wishes-

Post Office Box 85800, San Diego, California 92138-9216 • Telephone (619) 453-4100
TWX 910 337 1283 SALK SD LJLA

THE SALK INSTITUTE

Robert Malone

Jan. 11, 1988

Liposome Delivery of RNA to Eukaryotic Cells in Vivo

1. mRNA can be mixed with DOTMA lipid, the mixture placed on Eukaryotic cells in tissue culture, the RNA delivered to those cells and subsequently translated.

2. Liposomes can be used for molecule delivery to cells (in Vivo) with access to the circulatory system following venous injection of the liposome/molecule mix.

3. Capped, translatable RNA can be synthesized in Vitro.

4. Non-hydrolysable RNA analog synthesis of short RNA species is currently possible, and generation of large RNA analog molecules will probably be possible within 5 to 10 years. These analogs would be analogous to the non-hydrolysable methyl-phosphonate DNA oligonucleotides currently available. (Discussion with Dr. J. Walder, Univ. of Iowa on 1-11-88

Therefore:

The in Vivo transferance of mRNA to cells and subsequent translation of that RNA to protein by the method of DOTMA transfer of mRNA or mRNA analog is currently feasable. This technique will circumvent the problems associated with transcriptional regulation which currently plague gene transfer research. It is a way to treat RNA as a drug for therapeutic administration to patients.

Signed and understood:

Mark S. Kaday, Ph.D.

Date:

1/11/88

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VICAL INC.

Appendix B

9373 TOWNE CENTRE DRIVE, SUITE 100, SAN DIEGO, CA 92121 PHONE: (619) 453-9900 FAX: (619) 453-55

March 7, 1989

Anita Kirkpatrick
Knobbe, Martens, Olson and Bear
1st Interstate Plaza, Suite 1250
401 B Street
San Diego Ca. 92101-4235

Dear Anita:

This is a disclosure for a patent application that I regard as extremely important, requiring rapid action, and perhaps necessitating a request for accelerated review by the patent examiner.

The authors on this application are P. Felgner, B. Malone and J. Wolff (University of Wisconsin). I will be sending several examples by early next week showing data that our formulations can result in expression of gene products from RNA and DNA after in vivo injection.

Let's discuss this as soon as possible.

Sincerely,

Philip L. Felgner

cc.
W. Goodspeed
M. Demski
K. Hostetler
B. Malone

Elaborate your ideas on how to make the RNA better by using circular, blocking the ends, using "stronger" flanking sequences etc.

In Vivo Polynucleotide Delivery

Summary

Efforts to develop methodology for functionally delivering polynucleotides into living cells have continued steadily over the past several years. Effective methods include the use of calcium phosphate, liposomes, retroviral vectors, reconstituted viral envelopes and electroporation. In addition, there are several procedures employing polycations such as polylysine, DEAE dextran and polyornithine. (1,2,3,4) Reports citing the use of some of this methodology for in vivo polynucleotide delivery have appeared, but no consistant, convincing results have emerged suggesting that polynucleotides could soon result in practical therapeutic agents.

There are sizable drug delivery problems associated with the use of polynucleotides as therapeutic agents. This is because the molecules are so large, hydrophilic and negatively charged; these three factors mitigate against their ability to pass through the lipophilic and negatively charged plasma membrane barrier. Furthermore, polynucleotides (particularly RNA) are susceptible to the action of hydrolytic enzymes (DNAse and RNAse) which can rapidly degrade and destroy their intrinsic activity.

This patent application discloses compositions of matter, and methodology showing functional delivery and expression of genetic material, DNA and RNA, after systemic administration in vivo. The ease with which we can demonstrate that this occurs and the robust in vivo responses exemplified are particularly notable. These results represent a pivotal breakthrough, suggesting many novel, practical therapeutic modalities which will be discussed.

One novel perspective that is elaborated in this patent application involves the development of a simple injectable polynucleotide formulation that results in <u>transient</u> protein expression in vivo. To benefit from this strategy patients would need to take repetitive treaments, much as they do today with conventional therapies. This approach differs markedly from conventional thinking in the gene therapy field which elaborates the use of heroic and relatively impractical strategies, such as whole body irradiation followed by bone marrow transplantation. This type of heroic treatment strategy seems attractive, at first look, because it suggests that a single treatment procedure might result in a perminent cure. However, many years of basic research into this type of treatment modality have clearly shown that the approach is laden with daunting practical problems and grave risks to the patients or to the offspring of the patients. Included among these problems is the concern that any treatment which can permantently correct a genetic disorder, can also potentially produce a different genetic defect which could prove lethal or even more difficult to correct. There are regulatory concerns related to whether this type of treatment might result in accellerated passage of genetic defects into the germ line, resulting in suffering to future generations. A treatment modality that is commonly discussed for genetic disorders involves the use of retroviruses. However, these agents have the potential to activate an oncogenes, resulting in cancer. Many other practical issues related to the use of retroviruses, including how to manufacture

high titer virus stocks and how to deliver large genes, are particularly discouraging. Irrespective of all of these scientific, legal and ethical questions, researchers have been unable to consistantly and efficiently obtain enough in vivo expression by any methods to cure a disorder by genetic means.

The kind of transient gene therapy (heretofore, refered to as TGT) exemplified in this patent application, offers the benefits inherent in promoting the body's own protein expression (versus continuous infusion of a genetically engineered protein by some external delivery mechanism) without the threat of lasting genetic damage or virus induced cancer. This type of therapeutic treatment modality is particularly attractive for those cases where intracellular protein expresion is neccessary, such as for functional replacement of a cell surface hormone receptor. External infusion of, for example, the recombinent LDL receptor would not be expected to be an effective treatment for hypercholesterolemia, because it would not get functionally inserted into the plasma membrane of the target cell. However, delivery of the gene by TGT to the target cell would result in elevated surface receptor. From what is known about the biology of hypercholesterolemia, elevated levels of cell surface LDL receptor into target cells would be expected to result in an improved therapeutic outcome for patients with the disease.

Applications:

Treatment strategies requiring delivery of cell surface receptors

It could be argued that there is no need to decipher methodology for functional in vivo delivery of genes. There is, after all, an established technology for the synthesis and large scale production of proteins, and proteins are the endproduct of gene expression. This logic obtains for many protein molecules which act extracellularly or interact with cell surface receptors, such as tissue plasminigen activator (TPA), growth hormone, insulin, interferon, granulocyte-macrophage colony stimulating factor (GMCSF), erythropoeitin (EPO) etc. Appropriate delivery of a functional cell surface receptor by systemic infusion presents a seemingly intractable drug delivery problem; how can a recombinant protein be functionally inserted into the plasma membrane of the target cell? The solution to this problem would result in therapeutic strategies for the treatment of cystic fibrosis, muscular distrophy (distrophin???), and hypercholesterolemia. However, the drug delivery problems associated with properly delivering a recombinant cell surface receptor to its target cell in the proper orientation to result in a functional receptor seem intractable.

When DNA or RNA coding for a cell surface receptor, is delivered intracellularly, the resulting protein can be efficiently and funtionally expressed on the target cell surface. If the problem of functional delivery of recominant cell surface receptors remains intractable, than the only way of approaching this therapeutic modality will be through gene delivery.

LDL Receptor Gene

Rational: A slight elevation in the production of this receptor in the liver of patients with elevated LDL will have significant therapeutic benefits. The market for this type of therapy is enormous. The manufacturers of recombinant proteins would not be unable to compete, because simply administering the recombinant protein would not get the receptor into the plasma membrane of the target cells. The receptor must be properly inserted into the membrane in order to exert its biological effect. It will not be necessary to regulate the level of receptor expression; the more expression the better. This will simplify the molecular biology.

Strategy: Prepare lipid/DNA complexes containing the LDL receptor gene and make repetitive I.V. injections. The lipid complexes will be taken up largely by the liver. Some of the complexes will be taken up by hepatocytes. The level of LDL receptor in the liver will increase gradually as the number of injections increases. Higher liver LDL receptor levels will lead to therapeutic lowering of LDL and cholesterol.

Vaccine development

The results exemplified in this application will suggest that any protein for which the gene can be obtained can be expressed

locally after systemic injection. It follows that any appropriate antigen which is a canditate for a subunit vaccine, could be delivered as the gene rather than the protein product. The Protein expression in vivo would result in an appropriate immune response. For cell surface antigens, such as, viral coat proteins (eg. HIV gp120) the antigen would be expressed on the surface of the target cell in the context of the MHC, which would be expected to result in a more appropriate, vigorous and realistic immune response. It is this factor that results in the more efficatious immune responses frequently observed with attenuated virus vaccines. Delivery of a single antigen gene by TGT would be much safer that attenuated viruses, which could result in disease if they are not attenuated enough.

There is an additional advantage of TGT which can be exploited during the vaccine development phase. One of the difficulties with vaccine development is the requirement to screen different structural varients of the antigen, for the optimal immune response. If the varient is derived from a recombinant source, the protein usually must be expressed and purified before it can be tested for antigenicity. This is a laborious and time consuming process. With in vitro mutagenesis it is possible to obtain and sequence numerous clones of a given antigen. If these antigen can be screened for antigenicity at the DNA or RNA level by TGT, the vaccine development program could be made to proceed much faster.

Treatment strategies requiring intracytoplasmic or intranuclear protein expression

Some proteins are known that are capable of regulating transcription by binding to specific promotor regions on nuclear DNA. Other proteins bind to RNA, regulating its degradation or transport from the nucleus. Proteins of this class must be delivered intracellularly for activity. Extracellular delivery of recombinant transcriptional or translational regulatory proteins would not be expected to have biological activity, but functional delivery of the DNA or RNA by TGT would be active. Proteins of this type that would benefit from TGT would be NEF, TAT, steroid receptor and the retinoid receptor.

AIDS Resistance Gene

Rational: Introducing the AIDS resistance gene into an AIDS patients T-cells will render his T-cells resistant to infection by the AIDS virus. This will improve the patients ability to mount a T-cell dependent immune response.

Strategy: A population of the AIDS patient's own T-cells could be isolated from the patients blood. These cells could be transfected in vitro and then reintroduced back into the patients blood. The virus resistant cells will have a selective advantage over the normal cells, and eventually repopulate the patients lymphatic system. This will increase the level of T-cells and improve the patients immune system. However, this strategy would not be expected to eradicate virus in the macrophage reservoir.

(Use the NEF gene for this or the soluble CD4 gene to prevent budding. And use messenger RNA systemic delivery to macrophages in addition to the excorporeal treatment strategy as proposed above.)

Treatment strategies requiring delivery and functional expression of missing or defective genes
[Jon Wolff]

Controlled, sustained delivery of peptides

Conventional drugs, as well as recombinant protein drugs, can benefit from controlled release devises. The purpose of the contolled release devise is to deliver drug over a longer time period, so that the number of doses required is reduced. This results in improvemements in patient convenience and complience. There are a wide variety of emerging technologies that are intended to acheive controlled release.

TGT can be used to obtain controlled delivery of therapeutic peptides. Regulated expression can be obtained by using suitable promoters. Growth hormone, insulin, interleukins, interferons, GMCSF, EPO etc.

The T7 polymerase gene can used in conjunction with a gene of interest to obtain longer duration of effect of TGT

Episomal DNA such as that obtained when the promoter region for the Epstein Bar virus can be used. This is a way to obtain expression from cells after many cell devisions, without risking unfavorable integration events that are common to retrovirus vectors.

Calcitonin Gene

Rational: Controlled release of calcitonin could be obtained if a calcitonin gene under the control of its own promoter could be functionally introduced into some site, such as liver or skin. Cancer patients with hypercalcemia would be an obvious initial patient population. This project would fit into our business plan.

Strategy: Prepare lipid/DNA complexes containing the calcitonin gene and make repetitive I.V. or sub cutaneous injections. The liver or skin will become sites of calcitonin production, leading to elevated systemic levels of calcitonin.

[[[[Jon,
Delivery of NGF to brain for Alheimer's disease]]]]

Other examples:

Thymidine Kinase Gene
Rational: Introduction of the thymidine kinase gene into the macrophage reservoir will render those cells more capable of phosphorylating AZT. This should overcome their resistance to AZT therapy, making AZT capable of eradicating the HIV reservoir in macrophages.
Strategy: Prepare lipid/DNA complexes containing the thymidine kinase gene and make repetitive I.V. injections. The lipid complexes will be taken up largely by the macrophage reservoir leading to elevated levels of thymidine kinase in the macrophages. This will render the AZT resistant cell subject to treatment with AZT.

Interferon Gene
Rational: Increased levels of localized interferon production in macrophages could render them more resistant to the consequences of HIV infection. While local levels of interferon would be high, the overall systemic levels would remain low, thereby avoiding the systemic toxic effects like those observed after recombinant interferon administration.
Strategy: Prepare lipid/DNA complexes containing the interferon gene and make repetitive I.V. injections. The lipid complexes will be taken up largely by the macrophage reservoir leading to elevated localized levels of interferon in the macrophages. This will render them less susceptible to HIV infection.

Diphtheria Toxin Gene with a Tissue Specific Enhancer
Rational: Intracellular expression of diphtheria toxin kills cells. A functional diphtheria toxin gene delivered to pancreatic cells could eradicate the entire pancreas. This strategy could be used as a treatment for pancreatic cancer. The patients would have no insurmountable difficulty surviving without a pancreas. The tissue specific enhancer would ensure that expression of diphtheria toxin would only occur in pancreatic cells.
Strategy: DNA/lipid complexes containing the diphtheria toxin gene under the control of a tissue specific enhancer would be introduced directly into a cannulated artery feeding the pancreas. The infusion would occur on some dosing schedule for as long as necessary to eradicate the pancreatic tissue.

None of the strategies discussed address the use of anti-sense polynucleotides for turning off the expression of specific genes. The lipid complexes would be expected to efficiently deliver this class of polynucleotide as well. A similar list of rational and strategies could be generated for taking advantage of these formulations.

The research plan is very straightforward and simple. It would involve the use of a suitable reporter gene whose product

can be conveniently and quantitatively measured in animal tissue when it is successfully expressed. Many good possibilities for this reporter gene are known and readily available. A panel of different DNA/lipid formulations can be manufactured. The formulations can be injected into animals and the level of reporter gene expression determined. In this way it will be possible to screen many different formulations for efficacy.

2/2/89 Mouse CAT mRNA

Appendix C

DNA LINEARIZED ~ PstI

260/280 = 1.6 [DNA] = 1.55 µg/µl × 600 µl = 930 µg

Do 4 reactions

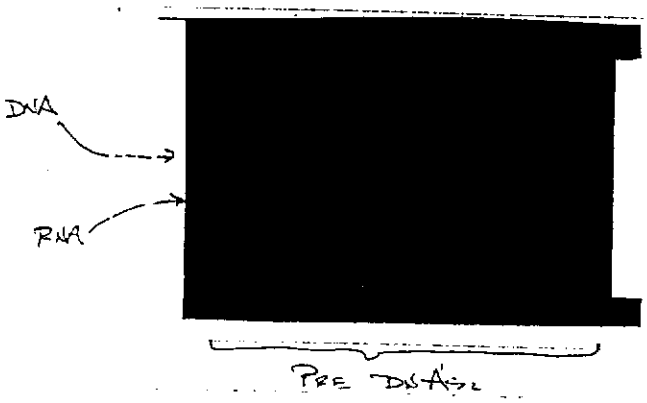
		#1	#2	#3	#4
DEP H_2O	158 µl	✓	✓	✓	✓
10× T7 Transcription Buffer	50 µl	✓	✓	✓	✓
100 mM ATP	5 µl	✓	–	–	–
100 mM UTP	5 µl	✓	–	–	–
100 µM GTP	2.5 µl	✓	–	–	–
100 mM CTP	5 µl	✓	–	–	–
10 mM CAT Antisense	25 µl	✓	✓	✓	✓
DNA (233 µg)	150 µl	✓	✓	✓	✓
RNAsin	25 µl	✓	✓	✓	✓
T7 RNA Pol	~~100~~ 75 µl	✓	✓	✓	✓

37°C, 1.5 h

THEN

H_2O	5 µl	✓
10× Buffer	30 µl	✓
RNAsin	15 µl	✓
RQ1 DNAse	220 µl	✓

15 min



8/27/89

10/5/89

3/14/89 TEST OF VARIOUS LIPOSOME/RNA TRANSFECTION CONDITIONS IN VIVO (CAT RNA PREPARED 3/7/89) (LUCIFERASE RNA PREPARED 3/10/89)

I.M. INJECTION:

GROUP #	GROUP TYPE	Vol OPTI-MEM	Vol 12½% SUCROSE	Vol SOLN D*	Vol CAT RNA	µg RNA	Vol H_2O
MUSCLE #1	DOTMA (-)	900 µl	—	100 µl	—	—	—
MUSCLE #2	CAT RNA (-)	879.1 µl	—	—	20.9 µl	15	100 µl
MUSCLE #3	DOTMA/RNA OPTI-MEM (+)	879.1 µl	—	100 µl	20.9 µl	15	—
MUSCLE #4	DOTMA/RNA SUCROSE (+)	—	879.1 µl	100 µl	20.9 µl	15	—
MUSCLE #5	HIDOTMA/RNA] SUCROSE (+)	—	→ 2 TUBES				

\# 5A 104.7 µl CAT RNA (75 µg)
395.3 µl 12.5% SUCROSE

\# 5B 37.5 µl 5 mg/mL LIPOFECTIN
462.5 µl 10.8% SUCROSE

*SOLUTION D = 375 µg/mL LIPOFECTIN IN DEP H_2O

I.V. TRANSFECTION FOR IN-SITU IMMUNOHISTOCHEM

- 195 µl CAT RNA (.1 mg) ADDED TO 28 mL OPTI-MEM IN 50 mL POLYSTYRENE CONICAL.
- "SOLUTION C" PACKAGED ⇒ 5 mg/mL LIPOFECTIN IN DEP H_2O

TAIL VEIN INJECTION:

TAIL VEIN (A) = 166.7 µl LUC RNA (300 µg)
583.3 µl 12.8% SUCROSE

(B) = 150 λ 5 mg/mL LIPOFECTIN (750 µg)
600 λ 12.5% SUCROSE

RNA CONTAINING SOLUTIONS FROZEN IMMEDIATELY IN LIQUID N_2.
SHIPPED ON DRY ICE.
LIPOFECTIN CONTAINING SOLUTIONS SHIPPED ON BLUE ICE.

INSTRUCTIONS AT LEFT PREPARED & SHIPPED TO S. WOLFF WITH REAGENTS.

8/29/89

VICAL INC.
9373 TOWNE CENTRE DRIVE, SUITE 100, SAN DIEGO, CA 92121 PHONE: (619) 453-9900 FAX: (619) 453-5885

Bob Hawley

TEST OF VARIOUS LIPOSOME/RNA TRANSFECTION CONDITIONS IN VIVO

3/14/89

MUSCLE INJECTIONS

Groups muscle #1 to muscle #5 should be processed as follows:

Muscle #1- Thaw, immediately add 100ul Solution D, inject into muscle
Muscle #2- Thaw, immediately inject into muscle
Muscle #3- Thaw, add 100ul Solution D, inject
Muscle #4- Thaw, add 100ul solution D, inject
Muscle #5- Thaw samples #5A and #5B, mix thouroughly, inject

TISSUE CULTURE TRANSFECTIONS

Thaw 50ml conical, add 70ul solution C, mix thouroughly, apply 4ml per 10cm plate for 8 hours, assay as you will.

TAIL VEIN INJECTION

Thaw tail vein A, add tail vein B and vortex (mix them in polystyrene tubes), inject as you will. Best to only mix as much as you need at a time and refreeze tail vein A between uses until it is consumed.

GOOD LUCK!!!

Phil and Bob

4/2/89 Test of Samples From Joni Wolfe

Sample #	30s Counts	Mg Equivalents
1	64.57	
2	21.550	
3	134.00	
4	92.92	~~16,078 pg~~ ~~1.6×10^{-8} g~~ ~~(.99 Mg)~~ 1.15 pg
5	50.65	
6	41.68	
7	18.36	
8	18.23	
9	17.800	

8/29/89

Jim Felgner
10/5/89

4/21/89 Analysis of Wolff Extracts.

Sample	Sample #	30s Counts	5/22	More Extracts, 37.54Q Acc 30sec
1	1	14.26	9A	12.51
2	2	14.20	9B	14.45
3	3	15.26	10A	12.56
4	4	14.73	10B	12.32
5	5	14.27	11A	12.43
6	6	14.40	11B	12.17
7	7	15.08	12A	12.46
8	8	14.54	12B	12.20
9	9	14.19	—Control	12.14
10	10	14.27		
11	11	14.45	H_2O + Buffer	12.17
12	12	14.56	H_2O Alone	12.05
13	13	14.30		
14	14	14.23		
15	1A	381.50		
16	1B	54.38		
17	1C	11.95		
18	2A	37.44		
19	2B	25.20		
20	2C	14.53		
21	3A	97.02		
22	3B	15.09		
23	3C	14.55		
1	4A	15.14		
2	4B	15.09		
3	4C	15.06		
4	5A	15.98		
5	5B	16.19		
6	5C	15.15		
7	6A	73.60		
8	6B	16.82		
9	6C	29.84		
10	7A	19.35		
11	7B	32.98		
12	7C	16.40		
13	8A ~~H_2O Buffer~~	15.13		
14	8B ~~H_2O Alone~~	14.91		
15	8C	51.19		

File Wolff 4/21 B

File Wolff 4/21 A

8/22

Jim Feely

Appendix D

TO: Jon Wolff
FROM: Robert Malone
RE: Analysis of Transfection Conditions

6/14/89

I am again sending nine sample groups (A-I) which have been divided into two aliquots due to differing storage and shipping requirements of the preparations. One will arrive at room temperature, the other will arrive frozen. Please store the room temperature aliquots at four degrees centigrade until use. Once again, we hope to compare the dose response and reproducability of polynucleotide delivery using these preparations.

Again, we would like to analyze these samples as follows

1. Warm the corresponding samples to 37C.
2. Mix corresponding samples by vortexing (ex: A1 and A2)
3. Immediately inject 100ul aliquots into the thigh muscle of 5-6 wk. old male Balb/C mice after surgically exposing the muscle fascia as previously performed. 2 mice/preparation (8 injections).
4. Close wound for 4 days and then sacrifice mice, dissect muscles, prepare tissue extracts and analyze for total mass of luciferase protein/muscle.
5. Please report data as both total light units/muscle and total mass of luciferase protein/muscle.

If you could record any observations which may impinge on the results as you are doing the injections, they might be helpful in understanding the origin of the variability.

Best of luck-

Robert Malone

FAX sent
6/14

VICAL UNKNOWNS
.100 ml 7/26/89

Vial CUT 6221.
CONTROL MUSC 18.970
19,290

A	1	18,260	
	2	17,980	
	3	19,590	F
	4	37,610	$\bar{X} = 22.91$
	5	27,250	5% Sucrose + LIPID
	6	21,280	
	7	19,570	
	8	21,280	
B	1	19,450	
	2	52,000	
Vial	3	33,140	E
	4	37,620	OPTI-MEM + LIPID
	5	18,820	$31.46 = \bar{X}$
	6	44,630	
	7	23,450	
	8	17,580	
C	1	41,890	H
	2	26,290	20% SUCROSE + LIPID
	3	23,000	$42.50 = \bar{X}$
	4	17,200	
	5	23,770	
	6	51,980	
	7	119,800	
	8	36,080	
D	1	21,490	B
	2	44,320	5% SUCROSE
	3	30,810	NO LIPID
	4	42,550	$32.89 = \bar{X}$
	5	20,770	
	6	52,970	
	7	22,100	
	8	28,170	
E	1	20,840	
	2	19,210	
	3	18,930	I
	4	18,070	- CONTROL (IB
	5	18,460	5% SUCROSE
	6	17,760	$\bar{X} = 18.42$
	7	16,840	
	8	17,230	
F	1	957,300	A
	2	30,520	OPTI-MEM
	3	1216.	NO LIPID
	4	219,700	$\bar{X} = 739.3$
	5	272,300	
	6	1848.	
	7	37,550	
	8	129,400	
G	1	25,970	G
	2	19,150	10% SUCROSE
	3	22,320	+ LIPID
	4	28,750	$\bar{X} = 27.58$
	5	29,200	
	6	25,810	
	7	44,760	
	8	24,730	
H	1	369,700	D
	2	27,210	20% SUCROSE
	3	19,550	NO LIPID
	4	26,030	$\bar{X} = 134.28$
	5	208,100	
	6	29,050	
	7	230,300	
	8	164,300	
		C 10% SUCROSE, NO LIPID	$\bar{X} = 161.32$
I	1	26,460	3 916,500
	2	48,280	6 78,900
	3	17,380	7 19,940
	4	164,00	8 19,080

group	code	DNA type	medium	sample#	counts	
A	F	pRSVL	Opti-MEM w/o lipid	1	957.3	588.85 mean
				2	30.5	630.2512 std. dev
				3	1216	
				4	219.7	
				5	272.3	
				6	1848	
				7	37.6	
				8	129.4	
B	D	pRSVL	Suc, 5% w/o lipid	1	21.5	32.8875 mean
				2	44.3	11.42644 std. dev.
				3	30.8	
				4	42.6	
				5	20.8	
				6	52.9	
				7	22.1	
				8	28.1	
C	I	pRSVL	Suc, 10% w/o lipid	1	26.5	161.325 mean
				2	48.3	289.1769 std. dev.
				3	17.4	
				4	164	
				5	916.5	
				6	78.9	
				7	19.9	
				8	19.1	
D	H	pRSVL	Suc, 20% w/o lipid	1	369.7	134.275 mean
				2	27.2	121.665 std. dev.
				3	19.6	
				4	26	
				5	208.1	
				6	29	
				7	230.3	
				8	164.3	

E	8	pRSVL	Opti-MEM w lipid	1	19.5	31.4625 mean
				2	57	13.34222 std. dev.
				3	33.1	
				4	37.6	
				5	18.8	
				6	44.6	
				7	23.5	
				8	17.6	
F	A	pRSVL	Suc, 5% w lipid	1	18.3	22.925 mean
				2	17.9	6.196319 std. dev.
				3	19.6	
				4	37.6	
				5	27.3	
				6	21.3	
				7	19.6	
				8	21.8	
G	G	pRSVL	Suc, 10% w lipid	1	25.9	27.575 mean
				2	19.1	7.199957 std. dev.
				3	22.3	
				4	28.8	
				5	29.2	
				6	25.8	
				7	44.8	
				8	24.7	
H	C	pRSVL	Suc, 20% w lipid	1	41.9	42.5 mean
				2	26.3	31.09952 std. dev.
				3	23	
				4	17.2	
				5	23.8	
				6	51.9	
				7	119.8	
				8	36.1	
I	E	pIBI31	Suc, 5%	1	20.8	18.425 mean
				2	19.2	1.161626 std. dev.
				3	18.9	
				4	18.1	
				5	18.5	
				6	17.8	
				7	16.9	
				8	17.2	

JP	sample#	code#	DNA type	medium	ul DNA	ul medium
A	A1	F1	pRSVL	Opti-MEM	30.8	569
B	B1	D1	pRSVL	Suc, 5%	30.8	569
C	C1	I1	pRSVL	Suc, 10%	30.8	569
D	D1	H1	pRSVL	Suc, 20%	30.8	569
E	E1	B1	pRSVL	Opti-MEM	30.8	569
F	F1	A1	pRSVL	Suc, 5%	30.8	569
G	G1	G1	pRSVL	Suc, 10%	30.8	569
H	H1	C1	pRSVL	Suc, 20%	30.8	569
I	I1	E1	pIBI31	Suc, 5%	46.9	553.1

			ul #28	medium	ul medium
A	A2	F2	0	Opti-MEM	600
B	B2	D2	0	Suc, 5%	600
C	C2	I2	0	Suc, 10%	600
D	D2	H2	0	Suc, 20%	600
E	E2	B2	8	Opti-MEM	592
F	F2	A2	8	Suc, 5%	592
G	G2	G2	8	Suc, 10%	592
H	H2	C2	8	Suc, 20%	592
I	I2	E2	0	Suc, 5%	600

RM #1	108	5	2.8	15	3.8	0.075	5.3	Opti-MEM	59
2	109	5	2.8	0	0	0.012	1	BCS, 10%	337
2	110	5	2.8	0	0	0.025	2	BCS, 10%	243
2	111	5	2.8	0	0	0.05	4	BCS, 10%	310
2	112	5	2.8	0	0	0.075	6	BCS, 10%	676
2	113	5	2.8	0	0	0.1	8	BCS 10%	1386
2	114	5	2.8	0	0	0.012	1	Opti-MEM	85
2	115	5	2.8	0	0	0.025	2	Opti-MEM	73
2	116	5	2.8	0	0	0.05	4	Opti-MEM	42
2	117	5	2.8	0	0	0.075	6	Opti-MEM	31
2	118	5	2.8	0	0	0.1	8	Opti-MEM	35
3	119	5	2.8	0	0	0.012	1	BCS, 10%	164
3	120	5	2.8	0	0	0.025	2	BCS, 10%	174
3	121	5	2.8	0	0	0.05	4	BCS, 10%	865
3	122	5	2.8	0	0	0.075	6	BCS, 10%	1981
3	123	5	2.8	0	0	0.1	8	BCS, 10%	2337
3	124	5	2.8	0	0	0.012	1	Opti-MEM	33
3	125	5	2.8	0	0	0.025	2	Opti-MEM	28
3	126	5	2.8	0	0	0.05	4	Opti-MEM	105
3	127	5	2.8	0	0	0.075	6	Opti-MEM	68
3	128	5	2.8	0	0	0.1	8	Opti-MEM	38.4
control	129	0	0	0	0	0	0	BCS, 10%	15
trol	130	0	0	0	0	0	0	BCS, 10%	15
trol	131	0	0	0	0	0	0	Opti-MEM	15
control	132	0	0	0	0	0	0	Opti-MEM	15

6/14/89

TO: Jon Wolff
FROM: Robert Malone
RE: Analysis of Transfection Conditions

I am again sending nine sample groups (A-I) which have been divided into two aliquots due to differing storage and shipping requirements of the preparations. One will arrive at room temperature, the other will arrive frozen. Please store the room temperature aliquots at four degrees centigrade until use. Once again, we hope to compare the dose response and reproducability of polynucleotide delivery using these preparations.

Again, we would like to analyze these samples as follows

1. Warm the corresponding samples to 37C.
2. Mix corresponding samples by vortexing (ex: A1 and A2)
3. Immediately inject 100ul aliquots into the thigh muscle of 5-6 wk. old male Balb/C mice after surgically exposing the muscle fascia as previously performed. 2 mice/preparation (8 injections).
4. Close wound for 4 days and then sacrifice mice, dissect muscles, prepare tissue extracts and analyze for total mass of luciferase protein/muscle.
5. Please report data as both total light units/muscle and total mass of luciferase protein/muscle.

If you could record any observations which may impinge on the results as you are doing the injections, they might be helpful in understanding the origin of the variability.

Best of luck-

Robert Malone

FAX sent
6/14

June 16, 1989

I. Alternative Lipid formulations (presently 25) for in vitro RNA transfection screening +/- serum; objective is to find formulations and transfection conditions that support transfection in the presence of serum

II. New DOTMA compound screening; Rosenthal series; John Silvius compounds

III. Supply formulations to Jon Wolff for in vivo screening in mouse muscle (supply Jon with both luciferase RNA and DNA, and the necessary lipid formulations; 9 groups per study, 8 injections per group; 1 study every 1 - 2 weeks).

IV. Repeat the NEF experiment

V. Prepare oligonucleotides for construction of Polio and EMC constructs; Jon's lab will make the constructs; Bob will do the in vitro screening; use the Vaccinia virus T7 to test the concept.

VI. Jon's lab will prepare the GP120 construct; British biotechnologies for a better GP120 (GP160 better?) construct that is easier to work with June 8 of Nature the (800)328-2400 (0865718817 telephone in England)

Sort out the differences in in vitro of DOTMA the toxicity
- Jon is repeating this

Time course of expression (in vitro [in process of repeating] and in vivo) How low would Bob go. Always urges people to look at cells every day. (Then we need to find out why is it so long expressed.)

India ink injections to evaluate variability

Sinbus virus constructs (I need to get the contact from Sondra so that we can make the right construct; does she have an infectious DNA construct)

Beta-gal problems

Tube A Room Temp leaked 3/4 gone

Tube C precipated slowly during injection

Tube D and E precipitated immediately (not injected)

First blinded RNA study:
DOTMA and free RNA were the same

CF grant application with Jon

55 minutes

Attorney Docket No. _____

LAW OFFICES
KNOBBE, MARTENS, OLSON & BEAR
A PARTNERSHIP INCLUDING PROFESSIONAL CORPORATIONS
PATENT, TRADEMARK AND COPYRIGHT CAUSES

Appendix F

NEWPORT BEACH

CABLE ADDRESS: CALPAT
TWX 910-595-1909 CALPATTHRN
TELEX 183513 CALPATT NBCH
TELECOPIER 714-760-9602

SAN DIEGO

1. BACKGROUND. Describe the field to which invention relates and explain what is wrong with the prior art. Make sure to give adequate background information to enable the reader to clearly appreciate the problems that existed prior to your invention.

2. DESCRIPTION. Write a detailed description of the invention referenced to sketches on the paper. If necessary, you may attach additional sheets, and you may refer to separate drawings or photographs by number. The signature information at the bottom of this page must appear on each added sheet, and on each separate drawing or photograph.

3. ADVANTAGES. List and explain the advantages of the invention in the order of their importance.

4. INVENTION RECORDS. Complete the "Record of Invention" form and attach it to this disclosure.

INVENTION DISCLOSURE

The invention relates to the methods of recombinant DNA technology as well as methods of delivering molecules to cells using liposomes. More particularly, the invention concerns the introduction of polynucleotides to cells by linking the polynucleotides to lipophilic compounds and formulating these modified polynucleotides in such a way as to allow them to enter cells. In addition, the invention concerns the stabilization of synthetic RNA molecules by chemically modifying the 5' and 3' ends of the synthetic RNA molecules.

In one aspect, the invention provides a method for synthesizing a stable polynucleotide which will recognize a specific cellular RNA by antisense base hybridization and then catalytically cleave that cellular RNA as reported by Haseloff and Gerlach , NATURE 334, 585 (1988). Prior art (supra) has demonstrated that activity of such catalytically cleaving polynucleotide sequences can be demonstrated in-vitro, however such sequences are likely to be very quickly degraded in-situ, and hence the utility of the prior art is greatly limited. In this aspect, the invention involves blocking the ends of the catalytic cleaving RNA by chemically synthesizing the terminal 5' and 3' sequences used for antisense recognition of the target as DNA, with the internal catalytic sequences chemically synthesized as RNA using standard solid state polynucleotide synthesis technology. Thus, the invention involves chemical synthesis of a 5'DNA/RNA/DNA3' chimeric molecule. It also involves any other chemical modification of the 5' and/or 3' ends of RNA molecules to protect those molecules from exonucleolytic degradation in-situ.

In another aspect, the invention involves a method for delivering polynucleotides (including polynucleotides such as those described above) across cell membranes by chemically modifying those polynucleotides by covalent attachment of lipophilic compounds to the polynucleotides so as to facilitate direct transfer of the polynucleotide across cell membranes or to allow the formulation of the polynucleotide into a preparation which will enable uptake of the polynucleotide into a cell. One example of such a formulation would be a liposome type preparation, and such liposomes may or may not contain cationic lipids to aid in the stable entrapment of the polynucleotide and/or the fusogenic

Robert Malone _____ PHILLIP FELGNER 6/1/89 Sheet No. 1 of 2
Inventor(s) Date 6/1/89

WITNESSED & UNDERSTOOD _____ _____
NAME (Not an Inventor) Date

WITNESSED & UNDERSTOOD _____ _____
NAME (Not an Inventor) Date

properties of the preparation. In one aspect, the covalent bonds linking the lipophilic compound to the polynucleotide may be hydrolyzable by cellular enzymes, and in another the bonds may not be subject to cellular hydrolysis.

The advantages of this invention are that it will enable the cellular delivery and stabilization of molecules which will specifically recognize and catalytically cleave RNA molecules. Such cleavage will then result in biological inactivation and rapid degradation of the target RNA molecule. This strategy can be used therapeutically to eliminate the expression of a protein associated with a disease process such as an oncogene protein (example: ras, myc, fos, src) or a protein involved in a critical phase of the life cycle of an infectious agent (such as the tat protein of HIV). Such a strategy may also prove useful in interfering with the disease produced by viruses with a RNA based phase in their life cycle (such as retroviruses and picornaviruses). This invention may also find use in defining the function of cellular proteins and RNAs by allowing the specific elimination of a single RNA species from the cell.

PHIL FELGNER 6/1/89

Sheet No. 2 of 2

Robert Malone
Inventor(s)

6/1/89
Date

WITNESSED & UNDERSTOOD
NAME (Not an Inventor) Date

WITNESSED & UNDERSTOOD
NAME (Not an Inventor) Date

Alternatively the invention involves synthesis of catalytic self-cleaving RNA moleucles containing 5' and 3' RNA stabilizing elements. These can be prepared entirely by chemical synthesis methods or by cloning the appropriate sequence into a plasmid vector suitable for in vitro transcription, to produce the final active product.

Polynucleotides do not normally associate spontaneously with lipid or surfactant based delivery systems or liposomes. In fact, the linear geometry of polynucleotide molecules can result in the exclusion of polynucleotide sequences from lipid vesicles. This results when the internal dimension of the vesicle is less than the longest dimension of the polynucleotide. Cationic lipid based delivery systems can overcome this problem by taking advantage of ionic interactions among the cationic vesicles and anionic groups on the polynucleotide. A minimum length of polynucleotide is required for this to occur stably; a sufficient number of cooperative ionic interactions is necessary to avoid displacement by water and salt. In order to overcome these problems, "Prosome-RNA" is a subject of this invention. Prosome-RNA contains lipophilic groups linked covalently to the 3' and/or 5' ends. Such molecules provide an additional mechanism for allowing interaction with lipid or surfactant based delivery systems.

VICAL INC
9373 TOWNE CENTRE DRIVE, SUITE 100, SAN DIEGO, CA 92121 PHONE: (619) 453-9900 FAX: (619) 453-58

TO: Wick Goodspeed
FROM: Robert Malone
RE: Resignation

8/1/89

I wish to resign from Vical effective August 25, 1989. I thank you for the opportunity to extend my previous work on cationic liposomal mRNA transfection which you have provided me. My primary reason for resigning at this time is that I am not interested in continuing to work under Phil Felgner and have him take credit for my intellectual contributions to the gene therapy project. Moreover, I have lost my respect for his managerial and scientific judgment. Thank you again for the opportunity to work here at Vical.