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CENTER FOR BIOLOGICS EVALUATION AND RESEARCH

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Twenty-eighth Meeting

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P A R T I C I P A N T S

Committee Members:

Daniel R. Salomon, M.D., Chairman

Richard E. Champlin, M.D.
Richard C. Mulligan, Ph.D.
W. Michael O'Fallon, Ph.D.
Edward A. Sausville, M.D., Ph.D.
Esperanza B. Papadopoulous, M.D.
W. French Anderson, M.D.
Xandra O. Breakefield, Ph.D.
Jeffrey S. Chamberlain, Ph.D.
Jon W. Gordon, M.D., Ph.D.
Carole B. Miller, M.D.
Bruce E. Torbett, Ph.D.

Guests/Guest Speakers:

Dale G. Ando, M.D.
Katherine A. High, M.D.
Donald Gay
Deborah Hurst, M.D.
John Levy
Louis Zumstein, Ph.D.
Richard J. Whitley, M.D.
Amy Patterson, M.D.
Estella Z. Jones, D.V.M.
Mercedes Serabian, M.S.

FDA Participants:

Kathryn C. Zoon, Ph.D.
Philip D. Noguchi, M.D.
Steven Bauer, Ph.D.
Carolyn Wilson, Ph.D.
Jay P. Siegel, M.D.
Karen D. Weiss, M.D.
Philippe Bishop, M.D.
Anne Pilaro, Ph.D.

C O N T E N T S

P R O C E E D I N G S

CHAIRMAN SALOMON: Can we get started? This morning, it is a meeting of the Biological Response Modifier Advisory Committee. If you're here for another advisory committee, you're in the wrong room. That is usually half the problem of being on the wrong airplane, I suppose.

Just a couple of little minor things. These are fancy new speaker microphones, but I think it is the same issue. After you speak, you have to push the button so the light goes on and off. Otherwise, what happens is two or three mics are on all at the same time and we drive this poor guy down there crazy, who seems to be on oxygen right now. I have a number of pleasures this morning to get started. One is to officially welcome Dr. Mulligan as a new member of the committee.

I also have the pleasure of welcoming back three very distinguished past members, Dr. French Anderson, Abbey Meyers, and the former chair of the committee, Dr. Miller. That was Julie. Sorry. Equally distinguished, who I fantasized was the chair. Anyway, it is a pleasure to have them all back. Thank you. Lastly, I would like to welcome all our guest speakers, Dr. Dale Ando, Dr. Katharine High, Dr. John Levy, Dr. Louis Zumstein, Dr. Donald Gay, Dr. Deborah Hurst, and Dr. Richard Whitley, who I think really provide a critical input of important new information, and I

appreciate that you could come here and join us today.

What I wanted to do, we're trying to zoom along a little bit, because Kathy needs to get out of here. But what I would like to do is just go around the table and introduce everybody, and just maybe give a sentence or two about what your area of interest is, because there are some new faces around the table. If we could start at the end.

DR. GORDON: I shouldn't say testing, one, two, three, but recount, one, two, three. I'm John Gordon from Mt. Sinai School of Medicine. I'm interested in germ line gene insertion.

DR. CHAMBERLAIN: Jeff Chamberlain from the University of Michigan. I work on gene therapy for muscular dystrophy.

DR. TORBETT: I'm Bruce Torbett from the Scripps Institute and we work on gene delivery to hemopoietic stem cells in animal models.

DR. BREAKEFIELD: I'm Xandra Breakefield. I work at Massachusetts General Hospital and I use herpes vectors for gene delivery to the nervous system.

DR. O'FALLON: Mike O'Fallon, biostatistician at the Mayo Clinic, and I don't know anything about the election.

DR. SAUSVILLE: I'm Ed Sausville from the National Cancer Institute Developmental Therapeutics Program and we

are interested in the preclinical valuation of drugs and biologics.

DR. PAPADOPOULOS: I'm Essie Papadopoulos. I'm an attending on the allogeneic bone marrow transplantation service at Memorial Sloan-Kettering Cancer Center in New York, and my interest is in the area of allogeneic BMT for treatment of acute and chronic leukemias, as well as adoptive immunotherapy for relapse and viral infections.

DR. CHAMPLIN: Richard Champlin, blood marrow transplantation from the M.D. Anderson Cancer Center.

CHAIRMAN SALOMON: Dan Solomon, Scripps Research Institute, organ transplantation, xenotransplantation, tissue engineering.

MS. DAPOLITO: Gail Dapolito, CBER, executive secretary for the committee. I would also like to introduce the committee management specialist, Ms. Roseanne Harvey, in the front row there. Thank you.

DR. MILLER: Carole Miller, Johns Hopkins University School of Medicine, leukemia and bone marrow transplant, clinical.

DR. ANDERSON: I am French Anderson, interested in various aspects of gene therapy, specifically targeted retroviral gene therapy, in utero gene therapy and stem cell gene therapy.

MS. MEYERS: Abbey Meyers, president of the

National Organization for Rare Disorders, and I was a former member of the RAC, and my interest is the informed consent document and patient protections.

DR. MULLIGAN: I'm Richard Mulligan from Harvard Medical School and I'm a gene transfer guy.

DR. PATTERSON: Amy Patterson, National Institutes of Health, Office of Biotechnology Activities, the office that supports the Recombinant DNA Advisory Committee.

DR. ZOON: Kathy Zoon. I'm the director of the Center for Biologics.

DR. WILSON: Carolyn Wilson, member of Division of Cellular and Gene Therapies, FDA, CBER.

DR. NOGUCHI: Phil Noguchi, director of the Division of Cell and Gene Therapy.

DR. SIEGEL: Jay Siegel, director of Office of Therapeutics and Center for Biologics.

CHAIRMAN SALOMON: Thank you, everybody. You got the button thing right, which is very good. This bodes well for the rest of the day. My next duty is to introduce Gail Dapolito to read the conflict of interest statement to initiate today's activities.

MS. DAPOLITO: Thank you, Dr. Salomon. This announcement is made part of the public record at this meeting of the Biological Response Modifiers Advisory Committee on November 16th and 17th. Pursuant to the

authority granted under the committee charter, the director of FDA's Center for Biologics Evaluation and Research has appointed Doctors French Anderson, Xandra Breakefield, Jeffrey Chamberlain, John Gordon, Carole Miller, Bruce Torbett and Ms. Abbey Meyers as temporary voting members. To determine if any conflicts of interest existed, the agency reviewed the submitted agenda and all financial interest reported by the meeting participants. As a result of this review, the following disclosures are being made. In accordance with 18 USC 208, Doctors Anderson, Breakefield, Chamberlain, Champlin, Mulligan Miller and Papadopoulos have been granted waivers which permit them to participate in the committee discussions. Dr. Salomon, Sausville, Torbett and Ms. Meyers have associations with firms that could be affected by the committee discussions.

However, in accordance with 18 USC 208 and 2635.502 of the standards of conduct, it has been determined that waivers or appearance determinations were not warranted for this meeting. In regards to FDA's invited guests, the agency has determined that the services of these guests are essential. The following interests are being made public to allow meeting participants to objectively evaluate any presentation and/or comments made by the guests.

Dr. Dale Ando is employed by Cell Genesis, Inc. Mr. Donald Gay is employed by Chiron Corp. Dr. Katharine

High has current financial interest in the topic. Dr. Deborah Hurst is employed by Chiron. Mr. John Levy is employed CTL Immunotherapies Corp. Dr. Amy Patterson is employed by the National Institutes of Health Office of Biotechnology Activities. Dr. Richard Whitley has current financial interest in the topic. Dr. Louis Zumstein is employed by Introgen Therapeutics, Inc. In the event that the discussions involve other products or firms not already on the agenda for which FDA's participants have a financial interest, the participants are aware of the need to exclude themselves from such involvement and their exclusion will be noted for the public record.

Additionally, while some of today's meeting participants may belong to different professional societies and organizations, they were invited today to express their own individual ideas and opinions, not necessarily those of any organizations with which they may be affiliated. With respect to all other meeting participants, we ask in the interest of fairness that you state your name, affiliation, and address any current financial involvement with any firm whose product you wish to comment upon.

Copies of the waivers addressed in this announcement are available by written request under the Freedom of Information Act. Just a couple of last administrative items. As a courtesy to the committee

discussions and to your neighbors in the audience, we ask that cell phones and pagers be set in the silent mode or turned off. Please step into the foyer if you would like to use your cellphone.

For the committee, there are slightly revised copies of the questions for committee discussion in the blue folders. They are little bit revised from what has been posted on the Web and what you received earlier, and the hotel has asked for a little bit of patience in getting the temperature equilibrated in the room.

Thanks.

CHAIRMAN SALOMON: The next item of business is actually both a pleasure and very important. I just have to say that the FDA has a very strong sense of gratitude to retiring members who have really worked hard, and the people being honored today have--I just started on this committee when they were in full swing and all three of them have made just remarkable contributions to the committee and I really respect them.

Kathy?

DR. ZOON: Thank you. We're going to get our exercise running around the table today. It really is a pleasure to be here this morning to honor two of our members who have been here and working with us so hard over the years, French Anderson and Carole Miller, and I just want to

express the center's gratitude, plus my personal gratitude, for all your efforts and your contributions in public policy and just good public health contributions.

I think the debt that the center owes you, not only for your participation in meetings such as this on very complex issues regarding gene therapies, cellular therapies, looking at bone marrow transplantation, whatever the topic, the contributions of these individuals have always been sound, have always been wise, and have always provided the balance and information for the agency to consider.

They have also participated in helping with our science programs. We have had our site visits and have done outstanding work in trying to help us improve the quality of our science at the FDA so we can do the very best job we can overseeing the products that we have. We thank you for that.

So, I would like to maybe ask Carole--if you could come up first--come on, Jay. Jay wants to say thank you, too. Carole, I just want to recognize you and thank you so much. I hope you do not mind if we call on you periodically to help us out. We would really appreciate it. Thank you.

DR. MILLER: Thank you very much.

DR. SIEGEL: I just want to say, Carole, that I reviewed what we did over the last several years in this committee, and virtually every product we considered,

Nupagen, Retexamab, intraleuken-11, the hematopoietic stem cell separators, you brought just some tremendous wisdom and help to us in dealing with them, and I want to express my great appreciation.

DR. ZOON: Next, French, can you come up? French and I have known each other for probably longer than either of us ever want to admit, and we always appreciate--at the very beginning of gene therapy, French came and educated us on the science of gene therapy and he has been helping to educate us ever since. Thank you, French, and I want to just thank you for everything, and be sure we will still call on you.

DR. ANDERSON: We should choreograph this better. I also want to add our thanks, not just for what you've done on this committee, but for what you've done in the community, in helping them understand what this committee does, what the agency does, our role in this process. It has been very important, and we will continue to hope that you continue in that role, as well as a consultant to us, and I know you have some regret, that we share, that your tenure here did not see gene therapy move into the area of approved medical products, but I think that great progress has been made and I think this is a fitting begin to symbolize, in fact, the importance of what happens here and of your contribution to it. Thank you again.

CHAIRMAN SALOMON: I'm working on Whoopi Goldberg to emcee the next award ceremony. I think you guys did great. Billy Crystal, I kind of like Whoopi, but--then it is my pleasure to get on with the scientific and discussion part of this meeting, which is, of course, why we're all here. The way we're going to begin session one is to basically deal with issues involved in the structural characterization of gene transfer vectors, and we will start with a series of discussions from the FDA and then from invited speakers, try and create some sort of scientific background for the discussions that will go on, and then, starting around 10:30, we will hope, we will begin the discussions with the committee--and again encourage active participation of the speakers, as well, who I consider really bringing a lot of expertise here.

There is a little time for questions after each talk, but what I would like to do, and I hope no one will interpret this as being impolite, is that I do not want to start the discussion of the questions, so the questions after the individual talks, I would like to sort of keep toward maybe highlighting an issue or clarifying something that came up, and I encourage you to ask those kinds of questions, but if it looks like we're veering off into a discussion of what we want to do at 10:30, I might interrupt and just put it off. I apologize in advance if I have to do

that. Anyway, I'm going to try and stay on time.

The first speaker is Stephen Bauer, from CBER. He spent part of yesterday trying to educate me on the construction of various different kinds of gene vectors, and I guess we'll find out how successful later today.

DR. BAUER: It is a pleasure to be here this morning. My goal during this talk is to set the stage for our discussions and our subsequent presenters about how we go about structural characterization of gene transfer vectors and what kind of information is important to get out of those characterizations, where we are with our current recommendations, what kind of information has come in that is new and has stimulated us to think about changes in our recommendations about what kinds of gene therapy or vector characterizations ought to be done.

The goal, of course, of structural characterization is to know that what you start with when you begin vector production is what you really think it is, and, of course, that at the end of the production process, that what you have is what you put into the system. If I could have the next slide.

To give you an idea of the complexity of this subject, this is a chart that shows our current active, as of a month ago, gene transfer INDs. There is about 190 of them and the majority are with three different vector types,

retrovirus, adenovirus and plasmid, but we're also getting into more, at least to us, exotic systems like poxvirus, herpesvirus, and, in terms of structure, these range on the order of three KB from plasmids up to as much as 300 KB for poxvirus.

And the systems that are used to produce these viruses or these vectors are divergent and they each come with their own properties, but they are all biological systems and inherent in biological systems is variability, which is why at CBER we look at the process, as well as the end product, in characterization of these gene transfer vectors. Some of these vector production systems are relatively straightforward, such as plasmid productions where you take a molecular clone of a plasmid, you put it into a bacteria, you let the bacteria propagate, you generate huge numbers of plasmids.

Others are much more complex, such as those used for adenovirus, in which you use intermediate plasmids which contain parts of the vector that will end up being the final construct. You transvect those into a cell line which mediates homologous recombination, which is a complex process, and then, using that which has been produced by the cell, you isolate a plaque and then propagate that.

Using a cell system that has the capacity to mediate homologous recombination, you're also producing a

product, so there is inherent variability in that sort of production system. But common to all of these, as I said, is--the principles that we use are you want to know in great detail the structure of the vector seed that you start with, and I think we have the most control of that. Then, at the end of the production process where you amplify to very high numbers of molecules, you want to know what you have produced at the end. If I could have the next slide.

The structural characterization, as I said, is to know that what you produced both at the construction phase of the vector and in the production phase, after you have made large numbers of molecules, that you actually get out the expected vector. You also can use the structural characterization to show whether or not the particular vector and production cell pair that you have is capable of making a reliable product in terms of its fidelity and structure.

Mostly the way that these kinds of characterizations are done are by restriction mapping, by PCR and by nucleotide sequencing, and each of these methods has advantages and disadvantages. Restriction mapping is a good way to look at the overall structure, but it is low-resolution in terms of small changes. Small changes can sneak by with that kind of analysis. PCR, it really depends on how you developed your assay, what you're going to look

for. It is very sensitive, but again you're limited to knowing what you are looking for. Sometimes you'll miss unexpected changes. Your target sequences of the primers are well-known, but what's in between, you do not know, at least if the size of the product is the same. Nucleotide sequencing we think of as the way to get the best answer for the complete structure of the vector.

When you have a population of vector molecules that are homogeneous, of course you know exactly what the structure is after sequencing, but the inherent variability of some biological production systems means that if you sequence, what are you sequencing and how representative of the real population of molecules that you're looking at is that sequence? If you sequence a clone, that is one clone. If you do PCR sequencing, you are probably not going to pick up something that is there at 10 percent or lower, and that has to be a clone to see it. If there is a variety of molecules in there, in the population you're looking at, you might miss things. How important is that? If I could have the next slide.

The structural characterization has an impact on three very important areas, which are safety, efficacy and, again, production of your vector. One of the primary issues related to structure of these viruses is whether or not replication-competent virus is produced. That is something

that we have very sensitive assays for some of our vector systems to detect, and I won't spend too much time on that subject today, but the other things that can change are vector backbone, the transgene itself can change, and our current recommendations have focused, at least early in product development, mostly on the transgene, the transcriptional control elements, and we have allowed product development to go forward with relatively less attention to the backbone.

We will discuss later today some of the pitfalls that might come along with that approach. But, if you alter the transgene, of course, you can easily imagine changing the safety of your product. But if you alter the vector backbone, what kind of things can happen? You can, for instance, introduce a new open reading frame that has a potential to make a protein that you didn't expect to be there. We might see an example of that later today.

Then, of course, you can also alter the efficacy of the product itself. You can have transcriptional control that is a lot less than what you expected, so you have less product produced. You can make a vector that just is not going to work as well as you had hoped. Then, again, looking at structural characterization is a good way to look at the robustness of the production process.

I am going to talk about a couple of examples of

what we have seen under our current guidelines with the information coming in that is kind of stimulating us to think maybe we should change the way that we do some of this characterization. If I could have the next slide.

There are at least two sources of genetic alterations. One is that what you start with isn't what you think it is, and this is surprisingly common in the world of molecular biology and vector construction. People swap plasmids and you think you know what you are getting, but you don't really always know that what you have is what you think it is. This illustrates the principle that, I think, for vector production, for what you're going to put into people, would like to have better characterization of the vector backbones that are being used. The intermediates are not as well-characterized as we often assume.

Then the other issue which is problematic and much more difficult to wrestle with is instability during vector manufacture, and, as I said, a lot of these systems are subject to inherent variability in which recombination rearrangements can occur during vector manufacture and also mutation as kind of a background rate of that, which we can't really do much about. There can be improvements in this area by alterations in the vector and the packaging cell line that you use that will help minimize these, but I think this is just something that is inherent in the systems

that we're using and it needs to be carefully thought about.

Our job was always to encourage increased capability to detect these sorts of things. Gene therapy seems to be--it's been around for 10 years but I think most of my colleagues and I look at it as a relatively new field still, and as we move towards treating patients who are younger, less seriously affected by disease, I think some of these things might become more important to look at. If I could have the next slide.

This is an illustration of this principle that you should know what you're starting with. This represents a surprise that has cropped up during analysis, kind of creeped up on us there. I just learned about how to do animation on PowerPoint, so you will have to forgive me. But, at any rate, adenoviruses are constructed, at least some of them, using different intermediate plasmids which you transvect on the cell line and allow homologous recombination to occur.

A relatively widely used vector system in which the E3 region has been thought to be deleted, it turns out that in the making of that particular shuttle plasmid, there was an insertion of unexpected sequence into that region of the shuttle plasmid. If your characterization is focusing on this area of the gene, you can begin clinical trials without realizing that you have an altered sequence in the

backbone of the vector.

This gene does knock out the E3 region, but surprisingly has homologies to salmon, human and drosophila sequences, introduces an open reading frame which does not have homology to any known protein, and the question can you detect expression, you can see it by RT-PCR, and whether or not there is protein expression from that is still an open question, at least as far as I know right now.

This illustrates the principle that, if you have not really completely characterized what you begin with, and then only later in the course of clinical development of a product you get around to complete characterization, you can be in for some surprises.

The next topic I will talk about is, I think, the more difficult issue of alteration during production of a vector. The idea is that if you have completely characterized a product, its intermediates, and you really have a good handle on the vector seed stock that you start with, you're going to put it into a production system and amplify tremendous amounts of molecules out of that. If I could have the next slide.

As I said, you can get during that process replication-competent viruses, and this has been observed with retrovirus and adenovirus production systems, but you can also get other kinds of mutations, and I think that is

an area that we have relatively less of a handle on compared to our relatively sensitive assays for replication-competent virus. If I could have the next slide.

Here, if you start with a well-characterized product and you put it into your packaging or your expansion system, you get still the possibility that mutants will arise during this process. For instance, in adenovirus, these are cell lines that will mediate homologous recombination and other kinds of recombination. If this occurrence happens and generates a replication-competent virus, we have assays, at least for adenovirus, that detect on the order of one-in-10-to-the-ninth particles that are produced that are replication-competent.

When we're talking about doses of 10-to-the-13th molecules, you could have 10,000 of these at that level of sensitivity administered to a patient. Does that come along with any toxicities? I think really we just don't know the answer to that, but I think it's important to think about. But that also indicates that the system that we're using not only will generate replication-competent virus, but also other kinds of changes, and improve--what we would like to know from the committee and our discussions is what kinds of improvements of structural characterization could we make that would help us get a better handle on these other kinds of alterations during vector production?

If this happens early in the production process, it is going to be relatively easy to detect, because it will be a higher proportion of the number of molecules. If it happens late, it is going to be more difficult to detect and then how much should we really worry about that is an important issue. If I could have the next slide, please.

Our current recommendations that are in the guidance to industry are that during an early phase of development of product, you sequence appropriate portions of the vector or restriction-map and look at protein. As I pointed out earlier, that has generally been taken to say let us look at the transgene and let's look at the transcription control regions and some of the flanking sequences, but it really hasn't meant that you can start in the clinic without knowing the entire--it means you can start in the clinic without knowing the entire structure.

The kinds of information I talked about before, with relatively incompletely characterized starting materials and intermediates, has stimulated us to think about what would we like to do to improve this--the characterization of these products. In discussions amongst our colleagues, we thought that perhaps the best approach would be to have, for vectors that are less than 40 KB, and this would include our three most commonly used virus classes or product classes, adenovirus, retrovirus, and

plasmid, that there be a complete sequence of the vector and the intermediates before initiation of phase one. This will be a point of discussion with the committee later.

Then, for those vectors which are larger than 40 KB, which I discussed, poxvirus, herpesvirus, perhaps other types, that we ask for sequencing of the intermediates and focus on the introduced regions, so that would be anything that is changed during vector production, in the backbone or the transgene, and then flanking regions. Exactly how much sequence is appropriate for a flanking region is also a question for discussion later this morning, and then that the sequence be completed before initiation of further trials in humans, expanded trials. If I could have the next slide.

I think the focus has mostly been on sequencing, and I think sequencing at the beginning of product characterization gives us the best confidence that we're starting with the material that we expect. I just described this proposal, but then the other issue deals with this instability or the inherent variability in biological production systems, and should we do more to look at product lots?

I have to point out that some of our sponsors sequence end-of-production vector, as well as at the beginning, so we have some focus just at the beginning. In

terms of looking at product lots, if you sequence, how representative is that sequence? I think it is important to know what the structure is, but if you have very low-level impurities in your vector preparation, is sequencing going to give you that information?

To my knowledge, nobody has sat down and sequenced 100 clones of a product lot to say well, this is the amount of variability that we have, and as I pointed out, it is a stochastic process, so one product lot is not necessarily going to be the same as the other, but we do have methods that will look at--to a lower resolution of product lot, such as PCR. The limitation with that is that you're looking at what you're predicting, what you see is dependent on the target, the primers that you use, and then restriction mapping, that has its stimulations.

But we would like to stimulate as much development in these areas as possible, so we know products we're developing are as characterized as possible. Next slide, please.

Another issue is what do you do with sequence that you have generated? If you do sequence an entire vector, adenovirus or other vector, 40 KB or less, what should be done with that? If you just submit to us a sequence without some analysis or sequence in a format that allows us to do analysis, that in itself is not informative.

At a minimum, we would like some discussion of these proposals that there be comparison to expected sequence, that there be analysis for open reading frames. That would be to look at this contingency that a mutation would introduce a new antigen into a vector backbone. Finally, comparisons to nucleotide and protein databases that are easy access, and are publicly available. This is an issue we would like some commentary this morning on. If I could have the next slide.

What do you do if you do find an unexpected sequence? Say you're at the beginning of your process and one of your shuttle plasmids not the expected sequence. You have invested nonetheless a lot of time and energy into producing that. One could start over again or say what additional experiments should be done to examine the effects of those unexpected sequences, and you could do studies where you look further at characterizing the vector in its altered form or its unexpected form and, as I mentioned earlier, look for open reading frames, whether they express RNA or protein, for instance.

You could decide preclinical studies to look at whether or not those particular changes really do have any effects, and you could add additional aspects or experiments in patient follow-up if there is sufficient rationale to go ahead with using that particular vector. If I can have the

next slide, please.

To summarize, I have kind of gone over what our current recommendations are, and shown that, in that context, we have seen unexpected material crop up in some of our vectors. And this has stimulated us to consider more rigorous structural characterization of products. We have made some proposals that we would like some feedback from the committee today on, and I think that our next speakers will give you a greater level of detail and insight into this particular phenomenon.

With that, I would like to thank the committee and the members of the audience and welcome any questions.

CHAIRMAN SALOMON: Thank you, Steve. Are there any questions to enhance clarity of this very clear presentation? Okay. I think there is no doubt that that is going to be a guide for us later this morning. We really will get back to those issues. Thank you, Steve.

The next speaker is Dr. Louis Zumstein, from Introgen Therapeutics, and he is going to talk about the identification and characterization of unexpected DNA found in an adenoviral vectors, and we really appreciate his willingness to come and share their experience in this issue with his vector construction.

DR. ZUMSTEIN: Thank you very much for the invitation. I am Lou Zumstein. I'm Director of Research at

Introgen Therapeutics and I have been asked to give a case history, if you will, of our DNA sequencing efforts for our new gene therapy product. In light of recent SEC regulations on fair disclosure, our lawyers insist that I show this slide and I would ask that you take note of the caution on forward-looking statements.

The basic premise of this talk is very much in agreement with what Dr. Bauer just said, which is that by sequencing data, the predicted origins and sequence of a vector needs to be considered to be tentative. The vector I am going to be talking about is RPRINGN201, less formally known as Ad5CMV-p53. I will be calling it Ad-p53 in this presentation.

We're developing this in cooperation with Aventis Pharmaceuticals for the treatment of cancer. The current clinical status of this vector is that it is in Phase III trials for advanced head and neck cancer. It is in Phase II trials for lung cancer. The predicted and demonstrated mode of action is to over-express p-53 protein in cancer cells and to cause those cells to die by inducing apoptosis.

A little bit more detailed background about how this vector is made and some general organization of the adenoviral vectors. This part of the virus, the expression cassette encoding p-53 and some flanking sequences, originates from a shuttle vector. The bulk of the rest of

the virus originates from a plasmid called PJM-17. The sequences in this plasmid trace back ultimately to an adenoviral variant called DL-309, and I will be going into that in a little more detail.

This virus is E1-deleted. E1 is essential for replication, so this is a replication and paired vector. E2, E3, and E4 are other groups of early genes. You should probably try to remember that the E3 region is not essential for viral replication. The L. genes are late proteins, primarily structural proteins. As is fairly common with gene therapeutics in development right now, Ad-p53 originated in academia and very shortly after the laboratories of Introgen were opened, Adp53 and the reagents used to make it came over from the laboratory of Dr. Jack Roth at the M.D. Anderson Cancer Center. The documentation we got with these reagents were restriction maps, not sequence.

I have shown you a map of the shuttle vector. I have noted that PJM-17 was derived from the Ad5DL-309 adenovirus. DL-309 was made in the laboratory of Tom Shank in the late 1970s. They did some manipulations to get rid of several restriction sites in that virus, and clearly their intent at this time was not to make a reagents to go into the clinics. This was a research reagent. Also keep in mind that DL-309 was selected as a viable virus. It

grows as well as wild-type Ad5, so the changes that happened to get rid of these restriction sites do not seem to have been deleterious.

At this stage, prior to clinical trials and before we started our sequencing program, we knew the historic building blocks of the virus. We knew there had been changes to several restriction sites that did not seem to be deleterious. And our program at this point was that, as the clinical program proceeded, as the number of patients treated got larger, as the trials got more important, our characterization of this virus was going to have to get more extensive and more sophisticated.

At this point, we also had a predicted sequence of the virus, but that sequence was based on sequences in the Genbank databases, not actual sequencing. The first sequencing we did was of a plasmid in which the P. 53 expression cassette had been cloned into. We did this literally within two months of opening the laboratory, manual sequencing. Again, this was prior to Phase I trials, and our conclusions from this was that the sequence of the expression cassette was as we had expected.

The new information we got was that the polylinker sequences in between the large pieces were now defined, for instance, the pieces of DNA between the CMV promoter and the P. 53 open reading frame. So, to give you a little more

detail, this is the expression cassette with some flanking adenovirus sequences blown up down here.

At this stage, we now knew the sequence of this region. At this point in time, we also had a number of QC assays in place to identify this virus, to show that it made p53 both biologically active and it killed cancer cells. The next piece of information we got on this virus was a paper from Bett, et al., in which they had sequenced a region in E3 where some of these restriction sites had been changed in the original DL-309 virus, and they noted that there was a two base pair deletion. They got rid of an expocyte down here, six-base deletion right here, they got rid of a restriction site, and a slightly more complicated event here that was a deletion of about 700 bases of E3 sequence and the insertion of about 650 bases of DNA, and I will come back to that in more detail.

The second piece of sequencing we did was GLP sequencing of the expression cassette and flanking sequences. We took a lot of clinical material, isolated viral DNA from that, cloned the expression cassette out into a sequencing plasmid, had that sequence to GOP standards, and we also sequenced straight from the viral DNA from within the expression cassette out in the adenovirus flanks.

Again, the expression cassette was as expected. We now knew the sequence of the polylinkers between the

expression cassette and adenoviral flanking sequences. We also found out that about 300 bases of the left adenoviral flank, that is, the sequences just to the left of the expression cassette, had more similarity to Ad2 than to Ad5. It is still very similar to Ad5 and in that region there are seven bases that agreed better with Ad2 than with Ad5. That region of the adenovirus is not expected to code for any proteins. The next piece of information we got on this virus was a paper by Gingras, et al., which sequenced the same region of DNA that I have been talking about in E3 and came up with a slightly different sequence than that of the Graham laboratory. At this point, the exact sequence of that insertion deletion event was unclear. We had restriction and PCR data of this region. We had been working on it, but the data we had did not differentiate between the two sequences.

The next step in our program to better characterize this virus as the clinical trials progress was to do full GLP sequencing of the entire virus prior to the initiation of pivotal trials. Again, after we sequenced the whole virus, the expression cassette was as expected. The E3 region sequence agreed with that of--from Cordova's laboratory, not from Frank Graham's lab, and aside from the E3 region, there were 23 discrepancies between the actual sequence of the virus and the sequences in the database.

To go into these discrepancies in a little more detail, 10 of them are expected to be either in non-coding regions or silent, nine are predicted to change amino acids in either known or predicted open reading frames, and four are expected to change the size of open reading frames. We believe that most, if not all, of these discrepancies are actually errors in the original Genbank sequence. The Ad5 sequence in Genbank is not the result of a large, well-coordinated sequencing program. It is a piecemeal compilation of sequence from a large number of laboratories.

In addition, when we have gone in and looked, the more recent literature agrees with the actual sequence of Ad-p53, not with the old Genbank sequence. Let me go into a little more detail about the changes that happened in E3. The top part of this map is the E3 region from wild-type Ad5 and predicted open reading frames. The bottom is the E3 region from Ad-p53 with predicted open reading frames. There is a 6 base pair deletion and a 6.7K open reading frame. It changes the expression level of this protein. It changes the number of glycocylated forms that one finds. As far as I know, the function of 6.7K is still unknown.

This is the insertion deletion event down here. 646 bases are inserted, where about 700 bases are deleted. The open reading frame for 14.5K is completely removed. 10.4K truncated. 18 amino acids are truncated off the end

and you get a fusion into sequences within this insertion, and you get a novel open reading frame that starts within this insertion and reads out into Ad5 sequences.

There's no protein similarity between this sequence and 14.7K because we're reading in a different frame here. 14.7K is not predicted to be made at all. Let me give you a little more detail about these proteins and what they do. 6.7K, I have run you through. 10.4K, 14.5 and 14.7K are all involved in preventing TNF alpha mediacytolisis. They are part of the mechanism Ad5 uses to avoid the host immune response.

The other point to make is that there are 27 amino acids fused onto 10.4 K. Neither those sequences, nor the novel open reading frame, have any significant similarity to known or predicted protein coding sequences in the databases.

What I'm going to do now is blow up just this region, this insertion/deletion of that 646 basis. That is down here. This is the novel open reading frame, 10.4K region from the left here. What do we actually know about this sequence? If you do a homology search with the sequence against Genbank, the only statistically significant similarity I pull up is a 92 percent identity to some sequences in the salmon prolactin 2 gene.

It is a 135 base pair region. That is right here.

That region in the prolactin II gene is downstream of the open reading frame. As far as I know, it is not predicted to encode any proteins. From Gingras, et al., we know that this DNA insert hybridizes to salmon DNA and not to human DNA. It is possible that the hybridization is just a result of this high homology region. From studies at Introgen and from Gingras, et al., we know that RT-PCR detects RNA made from this region and northern blots do not.

Northern blots are a fairly insensitive assay for RNA. RT-PCR is a very sensitive assay. Our guess at this time is that the transcript that is detected is a low abundance one. The only thing we don't know from RT-PCR is which strand is being transcribed here.

To summarize, we have now sequenced the whole virus prior to the initiation of pivotal trials. E1 deletion, p53 expression cassette--there was a two base pair deletion down in this part of the virus and a six base pair deletion here, but we also had a more complicated insertion/deletion of that, which resulted in some novel open reading frames. I will stop there and take any questions. Thank you.

CHAIRMAN SALOMON: Thank you very much, another very clear presentation. For the non-gene vector experts in the audience, can you explain why all of a sudden there is salmon DNA and in a vector you're constructing?

DR. ZUMSTEIN: Sure. First, I'm not entirely convinced it is salmon DNA. There is a high degree of similarity between that DNA and salmon sequences. It is not identical. Beyond that, there is speculation in both Bett, et al. and Gingras, et al. that that DNA originated from the transvection process.

When dl309 was made, they were trying to get rid of restriction sites, so they would cut the end of viral DNA, do a limited ligation and transvect that back in, basically looking for viruses that have lost some sites. It was typical at the time that when you do transvection, you use salmon DNA as a carrier. Whether they actually did that or not, I do not know.

The hypothesis from Gingras, et al. and Bett, et al. is that that carrier DNA got incorporated in as a result of an illegitimate recombination of that, if you will. Beyond that, I don't know where it came from.

CHAIRMAN SALOMON: Thank you. I guess the point I wanted to make, and we'll get to it later, is that one of the things to consider is, sort of, guidelines during the construction of these sort of vectors, and too, what additional products are being added to them, much along the way that we have looked at it in cell-transplantation and tissue engineering and xeno-transplantation.

All of a sudden, somebody adds fetal calf serum or

a co-culture with the a xeno line or something like that. The same thing, in a way, is happening in the construction of these vectors. You decide you have extra salmon DNA in a carrier and it gets incorporated in a vector.

DR. ZUMSTEIN: Certainly, when these regions were originally made, there was not the anticipation that they would end up in the clinic.

DR. MULLIGAN: This is off the topic, but what do you do about the p53 sequencing? One of the issues we will get into is, what amount of mutation of the coding sequence is actually going to be of issue? That will, of course, depend upon on what the protein is. I am kind of curious in the case of p53. First, what is your thinking in terms of what is acceptable in terms of having mutant p53 in your virus? And second, how did you go through the thinking of how to test for a one percent or a 10 percent mutation or something?

DR. ZUMSTEIN: Both of those questions are very good and very much out of my league. I think I'm going to pass on those. They seem to be topics, actually, for the committee.

DR. MULLIGAN: The answer is you have not, in your group, addressed this question?

DR. ZUMSTEIN: No, that is not the answer. The answer is that it is personally out of my expertise.

CHAIRMAN SALOMON: I think one of the things that is--they agreed to come and present this as a case history, and what we definitely do not want to give anyone the impression of is that Introgen or any other product going into phase III is part of the discussion today. I think it is very appropriate not to get into the specifics of that. We will deal with everything in general.

DR. ZUMSTEIN: We have certainly been in detailed discussions with the FDA, and they know exactly what we're doing and what we have.

DR. NOGUCHI: The questions that you raised were also discussed with Dr. Roth's original constructs, and there was actually a fairly extensive discussion about mutations in p53 and how one would detect these low-level mutations. As you correctly point out, p53 can mutate rather rapidly. I am not sure that we really came to a total resolution of the issue. I do know that that has been discussed both within FDA and publicly, at the rack, very extensively. It was about six or seven years ago.

CHAIRMAN SALOMON: Dr. Breakefield and then Dr. Anderson.

DR. BREAKEFIELD: I just wondered if you have done any in vitro transcription translation just to see that in an in vitro system--if you can make a protein?

DR. ZUMSTEIN: No, we have not done that. We have

certainly been discussing whether and how to go looking, to see if a protein was made off of this, but we have not done in vitro transcription translations. I think our first step would be to see which strand is being transcribed there, and those experiments are in progress.

CHAIRMAN SALOMON: That is a very good question. We will come back to that one, specifically, in question five of the discussion. Dr. Anderson.

DR. ANDERSON: Just for purposes of historical accuracy, when this vector was built, it was planned to go into patients, not by Introgen, but in terms of the discussion of the next two days, this reflects the fact that academic investigators are not as versed in all of the issues having to do with clinical trials that a large pharma is. But this vector, as built, was planned to go into patients.

DR. ZUMSTEIN: So, what I was saying was that ad5, dl309, when constructed in Tom Shank's laboratory--certainly was intended to go into the clinic?

DR. ANDERSON: You're absolutely right, yes.

CHAIRMAN SALOMON: Excellent. Thank you very much. The third speaker this morning is Jeffrey Chamberlain, University of Michigan Medical School. And he's going to talk about the instability of mini-adenovirus vectors.

DR. CHAMBERLAIN: Well, I would like to give an overview of a slightly different type of adenoviral vector system, in order to raise some questions about how we may want to consider making recommendations in the characterization of the system.

My laboratory, and a number of others, are interested in the development of so-called helper dependent adenoviral vectors, also known by various other names, such as gutted adenoviral vectors, or gutless adenoviral vectors. This system is unique from a conventional adenoviral vector system in several ways, in that a so-called gutted or gutless adenoviral vector is the end product that is being sought. This is a vector that does not contain any coding regions from the adenovirus itself. However, these vectors, at least with current technology, cannot be grown except in the presence of a more conventional adenoviral vector that functions as a helper virus. As a result, the system requires repetitive growth, where a particular packaging cell line is producing both the so-called gutted and the helper adenoviruses together.

I think this raises some additional issues. In particular, there is the potential in the system to encounter rearrangements, not so much by a conventional mechanism that you might see with a single adenoviral vector, but by homologous or non-homologous recombination

between the two vectors that are present in replicating in the same cell line. I would like to just give a little background on this system, and then point out some problems that we did encounter early on in the development of the system that may be instructive in terms of trying to figure out what types of requirements to impose on these types of vectors.

So, just to give a little background on how this system is used--there are several different versions of it in use in a couple of different labs. I think most laboratories today have a starting product of a plasmid-based vector, that contains small portions of the adenoviral genome, in particular, the left and the right inverted terminal repeat, as well as a packaging signal, and then a therapeutic gene expression cassette cloned into that plasmid vector.

Certainly, in my laboratory, we do all of our experiments starting with super-coiled plasmid preps. This is then co-transvected into a packaging cell line, together with one form of adenoviral DNA. Again, different laboratories use different forms of the helper. Some use co-infection, with a packaged adenovirus vector. We prefer to use either cloned adenoviral helper DNA in the form of a plasmid, or a purified protease treated in fetal chloroform extracted helper viral genomic DNA sequence.

In any case, once these are transvected into the packaging cell line, the helper virus produces all the adenoviral proteins needed in-trans for replication and packaging of your gutted vector, and these are both released from the cells and can be separated from each other in various methods. There are genetic selection methods and there are also physical methods, usually involving cesium chloride gradient centrifugation.

This system has some inherent inefficiencies, in that you're starting with a cloned version of these vectors, which typically do not replicate very well. Laboratories that are using this type of a system generally have to do a number of serial passages in order to slowly increase the titre of their gutted adenoviral vector. Here is an example from an early experiment we did, measuring titre by expression of a reporter gene. You can see that we would typically have to go out five or six serial passages, where we're growing both the gutted and the helper virus together in the same cell lines, before we would achieve a high-titre stock of the gutted virus, which was the goal of these studies.

Now, there has been a number of modifications to this system, where the number of serial passages can be reduced considerably, down to three or four even. However, I am not aware of any system where a person has been able to

do this by a simple co-transvection and immediately obtain a sufficient yield of a gutted virus to be useful without doing subsequent serial passages.

There are a variety of tricks that different labs have introduced to try to speed up the rate of growth, and to select against the so-called helper virus in this system. The most commonly used one is--takes advantage of Cre-lox mediated recombination. In this case, this is a system originally developed in Frank Graham's lab--the packaging signal of the helper virus is flanked by inserted lox p-sites. Then the adenovirus packaging cell that's used expresses the Cre recombinase. By co-transvecting your gutted virus DNA with your helper virus DNA, you can get Cre-mediated excision of the packaging signal of the helper virus. This serves a couple of purposes.

One, it tends to render the helper virus unpackageable by virtue of removal of that packaging sequence, yet the remainder of the helper virus sequence is still able to replicate and produce all the viral proteins you needed in-trans to replicate and package your gutted adenoviral vector. As a result, when the system is working well, the lysates prepared from these cells are tremendously enriched for the so-called gutted adenoviral vector, although inevitably there are still small trace amounts of contamination by the helper virus.

Typically, at the end of a preparation, one might want to do some further purification on cesium chloride. I apologize, this was not a particularly good gradient, but it was a convenient one that I was able to throw into this presentation.

Here is an example where one has a fair amount of residual contamination by the helper virus. This is the gutted virus here, and they're different sizes, typically, so they're resolved by a difference in their buoyant density on cesium chloride gradients. You can do multiple gradients, if you need to, to get fairly homogeneous preparations of your gutted virus. One of the reasons I illustrate this is to point out a couple of things.

One, even under the best of circumstances, there's generally at least .1 percent, if not higher, levels of the helper virus. Secondly, particularly with better gradients than I have shown here, which are not that difficult to achieve, you can get a reasonable feel for what types of viral genomes may be present in your mixture by potentially observing the presence of additional or unexpected bands or bands that might be migrating at a buoyant density different than what one would predict from their particular vector. That is a point I will come back to in a little bit.

Let me talk a little bit to some of the issues that might be involved in analyzing a preparation of gutted

adenoviral vectors. As I mentioned, I think essentially all laboratories that are using this system start with a cloned plasmid vector, such that your entire gutted or helper dependent adenoviral vector has been cloned into an E. coli plasmid vector and, as a result, it is really a fairly straightforward--one has a straightforward ability to go in and restriction map and sequence completely the gutted vector genome.

I think it is important that people also keep in mind the structure of their helper virus, since you're growing these two vectors together in the same cell and there is the potential for recombination or rearrangement, either between these two vectors or in one or the other of them. It is important that you know the exact nature of your starting helper virus. At least in the methods that we're using it my laboratory now--I mentioned that we use cloned helper virus DNAs where the entire helper virus is cloned into a plasmid. And again, it is a fairly simple matter, although somewhat expensive, to go through and restriction map and completely sequence the helper virus, as well.

I think an important issue in starting to characterize preparations of these viruses, is that you have very accurate titres of both the gutted and the helper adenovirus, so that you can make sure that what you are

seeing is what you really have there. I would suggest that before this technology is really going to be useful in the clinic, the efficiency of the growth of these vectors needs to be taken to the point where one can effectively generate a seed stock of the gutted adenoviral vector that will allow subsequent analysis of the initial preparation of the virus. What I'm trying to do here is to contrast that with the starting material, which is a plasmid vector that is subsequently converted into a viral vector, and that viral seed stock should be subsequently verified, prior to doing the individual preps that would then go into some sort of clinical trial.

The one question that we may want to address a little bit is at which individual stage is one going to require verification of the ultimate sequence? I think it is a simple matter to make sure that the plasmid you're transvecting into your plasmid cell line are highly verified and sequenced. When you get to the level of a seed stock, you're starting to deal with smaller and smaller quantities of these materials. And one may ask is it important to sequence off of the seed stock also.

I think it is a relatively simple matter to at least verify the density of these particles by doing cesium chloride gradients and to do some restriction analysis of the seed stock, but we may wish to consider whether it were

the seed stock itself or, perhaps, a sample preparation from that seed stock should be further analyzed. Probably, I would suggest by restriction analysis and biological evaluation. I think it is an open question whether additional sequencing might be required at that time.

Here's just an example of a generic gutted adenoviral vector, one of the ones we're interested in in my laboratory. I just want to point out a couple of things. Again, this is a conventional plasmid backbone. We usually use blue scrip from Strategene. It contains a CDNA cassette, a gene regulatory element. Often, you need a stuffer fragment to bring this up to an efficient size for packaging. It is important that one is aware of the nature of their stuffer fragment and what the sequences in there are.

And then, the only part of this vector that is derived from adenovirus, again, is a packaging signal and the left and the right inverted terminal repeat. Different versions of these vectors have a different size from these regions of adenovirus. It works well with a region as small as 600 base pairs. Some labs have taken that up to about two KB.

What are the requirements that I think are necessary to get this to be a viable system? One, you need a very stable vector backbone. It would be nice to have

something that is well sequenced and characterized, that enables cassette cloning capabilities, such that minimal modifications could be made to the vector. And that everything can be done either by homologous recombination, E. coli, or direct cloning, without the necessary, somewhat dirtiness of recombination of mammalian cells. You need to be able to derive a consistent, predictable structure from these vector backbones. You need very efficient and well characterized packaging cell lines that are able to grow these vectors to high titres without generating a replication competent adenovirus.

Again, you are going to need a well optimized helper virus that can support high titre growth of the gutted virus, preferably one that is self-disabling in order to minimize contamination of the preparations without generating RCA virus. And something that preferably gives minimal, if no gene expression in vivo, to prevent side effects from the helper virus. A problem I think of has plagued a lot of labs with this system is to be able to put all of this together and get very efficient, large scale growth of these vectors.

Let me talk about some of the issues in terms of being able to characterize these things. One is the issue of titreing of a gutted adenoviral vector. I think it is important that one is able to clearly define exactly how

much of this vector they have prepared, in order to start analyzing perhaps trace contaminants or impurities. A problem that has plagued a lot of labs is these gutted vectors are incapable of forming plaques, which used to be sort of a conventional assay.

You can determine particle counts, but you have to account for how much helper contamination is in there. You can put reporter genes into these things to look at transducing units, but preferably, I think you really want to go right at how many vector genomes you have by using some sort of quantitative PCR method, and this is a method that can be used to quantify both the helper and gutted virus, and is very useful in guiding the growth, but also assessing the purity of your preparations.

One question that is a little less clear, is what is the ratio of these different titreing units? I will show you an example of that, with just a sample prep from my own laboratory, where we grew up some gutted adenoviral vector and we assayed the titre by using a Taqman assay to do quantitative realtime PCR and got a titre a little over two-times-ten-to-the-ninth. We had a reporter gene in this particular vector and when we determined titre by tranducing that into 293 cells, the titre was about tenfold lower--excuse me, this is the helper virus I'm quantifying here. When we tried to quantify the amount of helper virus by

doing a plaque assay, as you can see, it was about another tenfold lower than that.

When people want to try to figure out exactly how much contamination they're dealing with, in terms of helper virus or perhaps other rearranged products, it is critical that you take into account exactly which assay you're using and what you expect to be able to detect with those various assays.

Some of the safety issues that will have to be dealt with are--some of these are similar to a conventional adenoviral vector, the toxicity of the vector. Obviously, a lot of preclinical testing needs to be done. Again, this issue of helper contamination, the structure and the stability of the helper virus, and then this question again of what type of quality control you want, in terms of when you assess it. Do you assess only the plasmid, your seed stocks or the final vector preparation?

I want to come back to this point of the serial passaging that is required to grow these vectors. I'm going to show you some problems that we ran into early on, before we started to get a better handle on this system. We initially encountered a very serious problem in growing these vectors, that we have largely overcome, but I think is still worthwhile making this point, because similar events could happen at a much lower efficiency. Then they might be

more difficult to characterize.

Here is an example of a helper virus and a gutted virus that we are growing at one time, that expressed the dystrophen CDNA, of interest to my laboratory, and we also had a reporter gene, the Lac Z gene that was driven under the control of its own promoter to help us titre this vector. Unfortunately, we had several relatively small regions of similarity or sequence homology between the gutted and the helper virus. In particular, the promoter that we had driving our reporter gene and the helper virus was the same promoter we were using to drive the Lac Z gene. It was relatively small, about 500 base pairs.

A second region of similarity was we had a 195 base pair polydelineation signal that was present in common between these vectors, and it was, in fact, located in two different places in the gutted adenoviral vector. It turned out when we grew these vectors together, we consistently recovered very high levels of rearranged products that, upon characterization turned out to be homologous recombinations that occurred in three different places. Either between these two polydelineation signals, these two polydelineation signals, or these promoter fragments.

Here is an example of the two most common ones where this homologous recombination event here between this promoter resulted in the transfer of the left inverted

terminal repeat and the packaging signal from the gutted adenoviral vector onto the right end of the helper virus, generating a helper virus that could no longer be selected against by Cre-lox mediated excision of the packaging signal. And instead, we now had a very robust, unselectable, robust growth that we could not select against this helper virus, that started contaminating our preparations at a very high level.

A second rearrangement did a similar thing, but resulted from homologous recombination between these polydelineation signals, that, again, brought the left inverted terminal repeat onto the right end of the virus and brought in a new packaging signal, such that this helper virus now started taking over the growth. One of the problems with this, is that these resulted in different sized vectors that no longer migrated at the density we predicted on cesium chloride gradients.

We were able to confirm these rearrangements by extracting DNA from these growths and doing southern blots with various sub-portions of the helper virus. I won't go into details, but on this particular prep here, we're missing a large portion of the right end. We have lost the right inverted terminal repeat, and we have a new fragment that hybridizes with the left inverted terminal repeat derived from the gutted adenoviral vector.

Again, let me come back to the question of cesium chloride gradients. In all of these cases, these rearranged products were immediately visible when we went to cesium chloride, because we started generating aberrantly migrating bands. However, we've also encountered some rearranged products that were smaller in size than normal, and which co-migrated with the gutted adenoviral vector. In which case, when we purified one of these particular viral bands to apparent homogeneity, and then we came back and started doing PCR assays, particularly the Taqman assay, together with southern blots and titreing of reporter genes, we clearly realized we had a mixture of co-migrating viral products in that band.

Not to leave you with too pessimistic of an outlook, when we had gone back and removed all of the sequences of homology between our gutted and helper viral vector, we are no longer seeing these rearranged products, but that does not necessarily mean one might not occasionally encounter those at a very low level. We feel that, interestingly, one of the things that is actually leading to the appearance and selection of these rearranged products is, in fact, our method that we used to try to select against the helper virus, which is Cre-recombinase, mediated disabling of the helper virus.

The real advantage of Cre-recombinase is that if

you do a gutted adenoviral preparation in the absence of Cre, such as we've shown here, you always have very high levels of the helper virus. And it's very difficult to get your gutted virus, shown in the blue-green here, to catch up to that. In the presence of Cre-recombinase, however, you have a constant selection against the helper virus, shown in yellow, and your gutted virus can accumulate to a level well over 100 times as concentrated as the helper virus.

However, we believe it is the very nature of the selection that allows us to end up with contaminations of rearranged helper viruses that have picked up a competent packaging signal. And, in all the cases we have characterized this, that competent packaging signal has always derived from the gutted adenoviral vector, although that is not to say that at some point one could not drive a competent packaging signal from the packaging cell line itself since a lot of those cell lines are E1-positive.

The other point I would make--even with these vectors that we worked with early on that had a propensity to recombine on us--when we did not grow those in the presence of Cre-recombinase selection, we never saw those rearranged products. This is something we were bringing out by the very nature of our attempt to select against helper virus.

Let me just close out with some suggestions from

my point of view. I think is critically important to quantify a gutted and helper by quantitative, real-time PCR methods. It is important to have a very firm grasp of the structure of both the gutted and the helper virus. I would propose that the starting plasmids from both of those be completely sequenced. At a minimum, there should be extensive restriction analysis and sequencing of ambiguous regions of the helper virus, if it's not possible to completely sequence your helper virus, although I would suggest that it should be possible to sequence a helper virus.

The question is, I think it is critical to have some sort of seed stock that can be verified, and the question is, will extensive restriction analysis and checking these preps on cesium chloride gradients be good enough, or would one want to require that the seed stock itself be re-sequenced for verification? I think I'll just stop there. Thanks.

CHAIRMAN SALOMON: Thank you. Again, another really nice talk and I appreciate everyone staying on time. It makes my job easy.

The question I have is, you talked about homologous recombination, if I understand right, and here, I'm getting out of my area of expertise, but I understand non-homologous recombination is also quite possible, and

that can occur between the helper virus and the gutted vector, but also with the packaging cell line. To the extent that that occurs, can you make some comment, again, with your expertise, just how often does non-homologous recombination occur? If so, how do you see that as having any implication, because given the fact that you're growing these things together in a packaging line, it almost seems like you'd have to sequence the lot every time you made one.

DR. CHAMBERLAIN: The most common example of that is perhaps the ability to occasionally pick up E1 positive sequences from the cell line. Although the rate of that occurring from a non-homologous event versus a homologous event is tremendously lower. I do not know the exact frequency, but there are examples of that occurring in the literature.

So, I do not know what to say in terms of how often that is going to happen. I guess, all I can say, with the limited experience we have doing a number of these growths, we have not observed a non-homologous recombination event that has cropped up in one of our gutted adenoviral vector preparations. That does not necessarily mean that it's never going to happen.

It probably will happen at some low efficiency, but I think that is certainly an issue that we want to consider--and why it's going to be important to characterize

these seed stocks as well as possible, and also, to try to develop methods that allow these vectors to be grown up with minimal rounds of serial passaging. I think the more times that you serial passage your two vectors together in the same cell line, the odds of a non-homologous recombination event are going to begin going up higher and higher.

CHAIRMAN SALOMON: Dr. Breakefield and then Dr. Sausville.

DR. BREAKEFIELD: I just had a question about the viral particles. You mentioned the possibility of titreing those, and I was wondering if, if they would really be resolved by the Cesium chloride, or whether they might give you a clue, for instance, if you had some background in that band. If you have, like, many more particles than you could account for by transducing units, you might suspect there wasn't the contamination, with empty particles or particles containing the smaller pieces of DNA.

DR. CHAMBERLAIN: Right. I agree. I think cesium chloride gradients can be quite useful in a number of different ways. I mean, we have typically been running these, just to help us purify the vector, although that is something that hopefully won't be required in the long-term. But, nonetheless, it does give you a good visual feel for what you have, and that gives you something to compare with.

I think you're right, if you simply take a viral

preparation and start doing PCR assays on that, you don't really know how accurate those numbers are, but you do get a very visual feel for what you have on the gradient, in terms of the number of bands you have, but even if you have a pure band, if your PCR assays are not giving your numbers that jive with what you've see on those gradients, obviously, you would have a problem there.

I think where that may become an issue, is if you get rearrangements that generate viral vectors that are essentially the same size, you are not going to resolve those on cesium chloride, but when you start doing quantitative PCR against select regions of the genome, the numbers may not match up all the way along the viral genome. If you test for the quantity of your transgene and then you test for the quantity of an inverted terminal repeat, or a sequence that is only present in the helper virus, those ratios should come out with what you're predicting or what you saw on the cesium chloride gradient. If you start to get rearrangements arising in the preparation, you're going to see altered ratios and they're not going to match up with what you might have predicted from looking at the cesium chloride. I think it's important to do both.

DR. BREAKEFIELD: So, do you think it is necessary, then, to actually measure numbers of viral particles or not, I guess, is my question?

DR. CHAMBERLAIN: Yes, I do.

DR. BREAKEFIELD: How do you do that?

DR. CHAMBERLAIN: I think the best way to do that is by quantitative PCR, to actually quantify the amount of vector DNA that you have in your preparation. I mean, if you're talking about taking a banded virus and also just measuring the optical density, to get a conventional viral particle count, yes, that should be done also.

In our experience, we generally get very compatible numbers with those two methods, but the PCR assay, I think, is a little more accurate. That is a good point. In fact, we do do that. We measure the viral particle count by conventional assay, and then we come in with several sets of quantitative PCR reactions, to pick up different regions of both the helper virus and the gutted virus, and make sure those numbers add up to what we saw on the particle count. If you had a rearrangement, then you would get a situation where those would not add up to what you had seen.

DR. SAUSVILLE: To pursue this line of questioning, my concern is, is there facile technology to not only detect the gutted virus plus the expected helper virus, plus a number of other things that one could conceive, or even not conceive rearranging, in relation to your particle count? Is there facile technology for that,

and would the incidence of this problem change from gene to gene, so that potentially you would have to reinvent this methodology, each time you chose a different target?

DR. CHAMBERLAIN: Well, I think the best method to use is really southern analysis. You can go in and take some of your viral preparation and digest it with a variety of different restriction enzymes, and hybridize it with different parts of your vectors, and make sure the restriction fragment pattern you see is identical to what you would predict from the sequenced material you're starting with. That is not necessarily the fastest and easiest method to do, and unfortunately, it takes, actually, a fair amount of a viral preparation to do that, which is, in fact, it can use up a reasonable amount of a seed lot to do an extensive restriction analysis like that.

Nonetheless, one could prepare a seed lot and then do subsequent growths from that, and use all of those subsequent growths for verification by southern analysis. The reason I would favor southern analysis is that it gives you a visual representation of the entire viral genome. You know, you can always go through it much more simply with less biological material, to do PCR assays across different regions of your different vectors. But if you don't know the nature of potential rearrangement, you're not necessarily going to pick it up by PCR, where southern

analysis should reveal all of the sequences present in your preparation, with perhaps the exception of a very low level contaminant. At the same time, I'm not sure there is any method that's going to pick up low level contaminants at a guaranteed--

DR. SAUSVILLE: Southern analysis--you really wouldn't expect to pick up things at less than a, say, 1.0, maybe .1 percent pressing it. You're still talking about a large number of potential particles or genomes that you're going to miss. Right?

DR. CHAMBERLAIN: Yes, that is true. But that is true of any viral vector, I think, so I think that is an issue that is certainly not unique to this system.

DR. O'FALLON: That kind of gets to my question, some of us were born at a time when 99.44 percent pure ivory soap was the epitome of perfection. I think I heard you say, and you just repeated it, that you had some of your gutted, which was 99 and--90 percent, 100 percent pure, and yet, we're talking about a lot of objects. Did I misunderstand you? You do have some that is that pure, but what is our target? Maybe that is our primary question here.

DR. CHAMBERLAIN: The purity I was talking about there, 99.9 percent pure, that is a purity of gutted viral vector versus the helper virus. I think the more critical

issue is what percentage of a rearranged product may be further contaminating those preparations. It is perhaps a separate issue, that we may not want to be dealing with here, is if one is going to do clinical trials with a gutted adenoviral vector, how much helper virus contamination would you allow to have in those mixtures? That is an issue of contamination of a known sequence.

DR. SAUSVILLE: I actually wouldn't be too worried about the helper virus. As you had described, there are robust methodologies to detect that. You know what you're going after. I must say I'm much concerned about the pieces I don't know might arise, with all the lines and colors that you had on your chart. I guess the bottom line is, in this technology, if there are 100 particles, can you say with certainty what fraction of them has the desired product, the helper virus that you think about? Then, is there a way of assessing what you cannot basically foresee or predict?

CHAIRMAN SALOMON: .1 percent of the 100 particles are, you know--

DR. SAUSVILLE: Are helper--

CHAIRMAN SALOMON: That is correct, but that is one point.

DR. SAUSVILLE: I'm not worried about the helper virus. We can look for the helper. I'm worried about what we don't expect.

DR. CHAMBERLAIN: Well, maybe to bring it into context, we can detect the helper virus contamination without too much difficulty. At that level of impurities, is detectable by southern analysis. I mean, I guess maybe what you're asking is, if the vector carrying you're therapeutic gene were to rearrange on you at a level of 0.1 percent, would you pick that up each and every time? With current technology, I would say probably not.

CHAIRMAN SALOMON: That is a good answer, and we will get into that issue in a minute. I just have one quick question, again. This may be a stupid question, but you're using 293, which has an E1 sequence, but your E1 sequence is in your helper virus. Why are you doing that? Why don't you just use a different packaging line?

DR. CHAMBERLAIN: I may have mis-spoken. Our helper virus is E1 deleted, but it contains the packaging signal, which I believe is present 293 cells. I may be wrong about that. Certainly the 293 cells are E1 positive. I assume they have the packaging signal, also. The helper virus is E1 negative, but we're trying to remove the packaging signal from the helper virus, and it can be picked up, in theory, from the cell line.

CHAIRMAN SALOMON: So, the point, though, is still, that the packaging line you're using has an extra gene not needed in the system that you have.

DR. CHAMBERLAIN: Well, more or less. 293 cells have been used for many years to grow E1-deleted adenoviral vectors, but at a very low frequency, you can pick up E1-positive replication-competent adenovirus due to homologous recombination with the E1 sequence that is in the cell line. There are some cell lines out there now that people have made different versions--inserted different versions of the E1 gene in there, to try to prevent that. Most of the cell lines are essentially unavailable to academic laboratories, though.

CHAIRMAN SALOMON: The direction I was going with those questions is, one of the things that's happening here, I think, is this tension at the nexus between an academic laboratory and Pharma. Everybody uses 293, so you're doing all these elegant, you know, Cre-lox changes and things in the helper virus, and yet we're all still using the same packaging line, which, at a certain point, probably is not the ideal packaging line for the system.

DR. CHAMBERLAIN: Right. I think that gets into a little different issue. There are alternate packaging cell lines that can be used, there are also other ways to select against RCA besides concerns of the E1 region; particularly, by using vectors that have additional modifications, such as deletions and the E2 region or the E4 region and things like that. I think there are very effective ways to select

against and to screen against the E1 positive viruses. I think a more significant concern now, is how to deal, though, with less predictable rearrangements.

CHAIRMAN SALOMON: Are there any other questions? Well, remarkably, we are at 10:10 a.m., exactly time for a break. I will see everyone back here in 10 minutes, please, and we will keep on going. Thank you.

[Recess.]

CHAIRMAN SALOMON: I am always reluctant to cut short these first breaks, since I know for a lot of people it is a nice opportunity to get together with people and of course, you're all discussing the FDA questions during the break.

MS. DAPOLITO: They shouldn't be.

CHAIRMAN SALOMON: Is that right? We're really not off time, because we actually started 10 minutes ago, is where I was going with that, but maybe not.

As we get started again, there is one more speaker. I would like to introduce Dr. John Levy from CTL ImmunoTherapies Corp. to talk about "The CMV Promoter is Copied as "Extra DNA" from DNA Vaccine Plasmids." And after that, then we will go into our discussion. Thank you.

MR. LEVY: Well, thank you. I'm the Quality Control Manager at CTL ImmunoTherapies Corp., and I have been invited here, I take it, to talk about extra DNA

sequences. That certainly fits the description of what we found in our product.

The identification of this, I will go into some of the issues related to that toward the end, but essentially, we're using plasmid DNA. We are not using the traditional viral vector systems. The DNA we're going to be using is going to be used as a polynucleotide vaccine. From the standpoint of how we put the vectors together, I would just talk about a couple cloning constructs. The backbone we chose to use as our starting point was a vector from invitrogen PVAX, which has apparently been engineered to become a very simplified, streamlined vector to contain reasonably desirable elements that could be used in these polynucleotide vaccines and, hopefully, not other elements that would be not particularly useful in vaccine research.

It contains a PMB1 origin replication, a CMV enhancer promoter region, bovine growth hormone polydelineation sequence, and a kanamycin resistance chain. We wanted, for the purposes of what we wanted to do for our vaccine, is express two genes simultaneously. I understand there are a number ways to do that to promoters. There are systems you can use that would involve differential splicing, but we chose use an IRES sequence as our way of simultaneously expressing a gene from the cap-dependent site upstream and the cap-independent site downstream of the IRES

sequence.

We obtained our IRES sequence from a commercially available vector called PIRES. Essentially, we put these two constructs together, cloning the IRES gene sequence into the multi-cloning site of the PVAX gene to make a hybrid vector PVYY1. You'll have to excuse the--I was learning to use animation the other night, too, and actually, me and John took this stuff off the presentation, but it persisted--the contamination of an animation sequence.

I should point out, as I get a few slides into this, I'm going to describe a small region called a sequence terminator that is a sequence found within the uppermost part of the IRES that is available commercially. And we did a little bit of analysis to determine what effect that has. Essentially, the bottom line is, when we make these plasmid vaccine preparations and run them out on gels, where the vector contains the PVAX backbone with an IRES sequence in it, and independent of the transgenes, we see as in either lanes B or C, the super coil DNA high mobility migration in the gel, but we see these other bands and we see them very persistently. They gave us pause when we first saw them, and the fact that they were 600 base pairs and a couple of sequences below that, led us to believe that we potentially had some double-stranded fractions of DNA in our preparations, but what they were, we weren't really sure.

What this slide is meant to represent is an early attempt to see if there's any way to get rid of the 600 base pair, and the two sequences below it are approximately 300 base pairs and 360 base pairs. In lane C, we're using a host strain that is fairly common in the field, DH5As. What we found is if we, instead, put our polynucleotide vaccine into a host strain DH10Bs in lane B, that we could just by doing that, consistently reduce the relative content of these bands about threefold.

The question is are these things related? Is the 600 base pair piece of DNA that we're getting in these vaccine preparations related to the 300 base pair and 360 base pair piece? Actually, when we run this out on a two percent augerose gel, run it quite a ways, it may not be visible from the slide, but the 300 base pair band actually is a doublet that migrates as a 280 base pair fraction and approximately a 310 base pair fraction. We cut it with an enzyme--well, I should probably say that, in looking at the literature, we just formed the idea that the most likely possibility of this is that they are what are described as replication intermediates in the literature.

There are a lot of papers out there where experimental replication intermediates are deliberately formed by putting two origin replications, basically facing right at each other, to form an active and silent origin

replication producing these fragments. There are some instances in the early literature that these were happening on PPR345 plasmids. Essentially, we went from that direction--that this was probably an origin of replication phenomenon.

If you look at a unique restriction site, about 130 base pairs down from the origin replication, and consider that a unique restriction site and digest these bands with this enzyme, ASC1, what it does is it actually cuts all of the bands and generates an accumulation of about 130 base pair common fragment, leading to the idea these are all a family of fragments, all originating from the same point. And, based on this enzyme and, at least in our plasmid, it is only represented in a position, which you would expect this behavior if it was occurring at the origin of replication.

We gel-isolated, and purified, cloned and sequenced the 600 base pair predominate fragment and aligned that to--our first best guess was it was something probably generated from our vector in the host strain, and the replication intermediate, or replication anomaly, has 100 percent homology match to a sequence within our vector. And that sequence is a sequence that would imply it starts with the origin of replication and extends through the entire CMV enhancer region and is terminated at approximately the CMV

enhancer/CMV promoter boundary.

In lieu of actually doing some genetic manipulations to the plasmid to address the question of can we get rid of this thing within the context of the arrangement of the genetic elements in the vector, we did some cell culture techniques to see if there were just some straightforward things we could do in the fermentation or production process that would attenuate a level of these replication intermediates. We noted that if you change the temperature of the fermentation production process, that lower temperatures--when you lower from 37, down to 35, down to 33, you get approximately a twofold reduction in the accumulation of these replication intermediates. In this case, it is in the DH10B cell lines, which themselves have already been threefold attenuated, in comparison to the DH5A cell line.

You can further affect the level of accumulation of these replication intermediates by choosing a time in your fermentation process that extends beyond a 12-hour time point. It seems that, at 12 hours, when your host cell strains are first coming out of their mid-log phase growth, that these replication intermediates are at their peak accumulation. They tend to, if you will, de-accumulate over time. If you go to 16 hours or 20 hours--it seems to tail off if you go out to 24 or 48 hours, that you can effect

about a two-to-threefold reduction on top of the reductions that you've seen already.

So, cell culture conditions can actually lead to some attenuation of this phenomenon. We wanted to study what this replication intermediate was, how it occurred and, if possible, how we could eliminate it within the confines of our construct. We performed a deletion analysis, since it is that the sequences mapped--two sequences in the CMV promoter originating from the start side of the origin of replication--we first decided to do a deletion analysis on the--some sections of the CMV promoter. And we have kind of an opportunistic deletion, one of those laboratory anomalies where you got, apparently, a star activity digestion of the enzyme that cut some portion of the IRES sequence out at this stage of the deletion analysis.

And what you see is that in an undeleted vector preparation, you can compare the replication intermediates that you see to the deleted. So if you delete out essentially two-thirds of the CMV enhancer and CMV promoter sequences, you effectively get rid of the predominate 600 base pair replication intermediate band, but retain the lower doublet, 280, 310 and 360 replication intermediate bands. And it is not until you actually delete further into the E-CMV IRES sequence, that you're actually seeing a complete elimination of all of the replication

intermediates.

From the standpoint of is this a strategy that would actually make a better vector, the problem, of course, is that you're actually having to delete out sequences that you need for function of your vector in the vaccine activity of the vector. We went back, and I know this seems a little bit backwards, but this is a deletion where we took the entire CMV promoter out. There are a couple of ASE1 sites. This ASE1 site was the one I used to map the upstream portion of the replication intermediates.

What we found was that if you consider our deletion that we just performed here, taking the entire CMV promoter out, you produce, actually, a new replication intermediate that would map now within the IRES sequence. If you take the original backbone vector, which PVAX represents a, essentially, IRES deletion, you do have still the approximately 600 base pair band that you see from the vector that has both the CMV and the IRES. However, in both cases, there is a tremendous attenuation of the accumulation of these species.

The opportunistic deletion that actually cut off a sequence within the IRES was something that we looked into a little further. We knew that in the sequencing of this vector--we have sequenced this vector in its entirety multiple times at master cell bank and production lot phases

to ensure its integrity throughout the process. We noticed that there is a sequence within the five-prime (ph.) region of IRES sequence that we could never completely sequence beyond in either direction.

Actually, this is a very dominant sequence termination site that actually became kind of a map site for alignment and context analysis, because you always knew where you were at by just watching where all of the context converged--was a sequence that is very C-rich, CT-rich. It is actually--this portion of the IRES maps to a portion in the wild-type strain of the virus where that sequence is just coming out of the poly-C track. So, this is a C-rich sequence within the traditionally-defined IRES domain.

It is very difficult to sequence beyond it. You can sequence through it, and then these big-dye terminator, cycle-sequencing reactions will fail after that in either direction. I speculated, well, this is a polymerase, a bacterial polymerase. I'm not sure if the wild-type polymerase is in--the host strains are having not necessarily the same problem, but at least the sequence is potentially giving them some biological response.

We simply just deleted out that sequence, this sequence right here, and we can see by simply doing that--this is our vaccine vector with the CMV and the IRES sequences in it. If you just take out the sequence

terminator, you get a tremendous attenuation in the accumulation of these replication intermediates, implying that there is some contribution of the IRES to the termination that is occurring on CMV and the IRES sequence.

If you just cut the sequence terminator, you get a tremendous attenuation in the accumulation of these replication intermediates, in ponderous some contribution of the IRES determination those occurring on CMV, considering what is known about termination interactions in host strains, considering that perhaps maybe the sequence is inciting something in the bacterial host strain that is leading to a biological response that maybe we would predict, and that potentially would be an interaction that is known as replication termination in the host strain, through proteins called tuss (ph.) and termination sites called terr (ph.), and that body of literature, that phenomenon of termination within the E. coli host genome is orientation dependant, so we figured by simply just turning the origin replication in the other direction, perhaps we would actually produce a plasmid vector, and these are meant to be circular, of course, produce a plasmid vector that would test the hypothesis of whether these are contrahelic cases that are binding the CMV promoter or whatever they may be, that there are operating in an orientation-dependant fashion.

And what we see is that our plasmid vaccine, here in line A is the comparison, simply by turning the origin replication in the other direction within this vaccine, there are no replication intermediates seen anymore, at least at the level, unloading--there are probably 40 micrograms of DNA loaded on these, we do not see anything in these lanes. Within the detection limits that augerose gel electrophoresis can give you, there is nothing that we can detect as far as existence of these species anymore.

Just as a note, there is another vector used traditionally or has been used traditionally in the DNA vaccine, PCDNA-3, and I think I put the map for it on there. I do not know if this necessarily has anything to do with it, but within this vector, there's an ampicillin resistance marker ribbon, kanamycin resistance, that does have a CMV promoter. You can put an IRES sequence in there if you like, but the origin of replication in this construct is running in the opposite direction of the CMV promoter, similar to the construct that we modified from our own laboratories.

On the last lane of that gel, I would just kind of reiterate what was in the last line of the last gel, is PCDNA-3 vector does not produce these replication intermediates, not even at low levels. We can say that our plasmid vectors do contain these sequences that result in

replication anomalies, replication intermediates, that the CMV enhancer promoter contains sequences that seem to synergize with demands within the IRES to produce more of these replication intermediates than you would see in plasmids that would just contain either one or the other of these sequences, and that replication anomalies--we have actually--I have not actually brought light to this--but we have actually seen replication anomalies in other plasmids with similar arrangements that do not necessarily have IRES sequences, but have sequences similar to this termination, the sequence termination or C-rich element within the IRES.

Deletion, obviously, is not always going to be a practical means in these vectors, if deletion means actually getting rid of the promoter enhancer sequences that you're going to need further down in your clinical work to actually express the genes that you need for your DNA vaccine, but that inversion of the origin replication apparently eliminates these replication intermediate anomalies and may reflect the orientation-dependant nature of how these things are actually produced.

Thank you very much, and I will be happy to answer any of your questions.

CHAIRMAN SALOMON: Thank you very much, another real interesting view of an alternative way of getting sequence anomalies in vector production. Any questions?

MS. MEYERS: Just as an overall perspective on this morning's presentation, the public, I think, has more understanding of pregnant chads than anything that was said here today. It is a whole new vocabulary. Since there are these problems and there are--these replication anomalies occur in a lot of different experiments, there seems to be production of abnormal viruses that have never appeared on Earth before. How do you throw them away? Do you throw them in the dump? Do you throw them down the drain? What do you do with these very different viruses?

MR. LEVY: Oftentimes--well, in the case of a double strand of PCDNA, DNA is exquisitely sensitive to common chemicals like acid, so autoclaving is a high enough heat to actually remove, not only viruses, but DNA, so there are some common sterilization techniques that laboratories always produce within the normal confines of actually developing production protocols or manufacturing protocols that should address actually eliminating these sequences.

MS. MEYERS: You destroy these viruses before they are thrown away?

MR. LEVY: If there is a proven or perceived safety risk, certainly I think someone would want to evaluate, you know, what the expediency for actually removing these entities are. In some cases, obviously, it is necessary to generate enough of these entities to study

them, to understand what they are. If one were to see something that was anomalous and immediately get rid of it, you might not actually ever know what it is that produced it.

In some cases, you would have to assess what the risk of the entity was and find ways to either safely study it or expediently get rid of it.

MS. MEYERS: You do keep some of it alive in your laboratory, maybe forever?

MR. LEVY: Well, in our case, these sequences are DNA. They are not viruses. On their own, they will not replicate. If you put them in a tube, frozen away in a freezer, presumably, if you came back in 100 years, in the cases from our studies, you would hope that even if you came back in 10 days, that they would still be there. In the case of viruses, though, if you freeze viruses away, there are ways of actually containing viruses over long periods of time.

I think it would be difficult to assess, you know, what the safety of each of the viruses is unless you had some idea of what it is you're actually working with.

MS. MEYERS: My understanding, though, is a lot of times these viruses that have been made up in these experiments, we really don't understand, because they've never existed before. Am I right?

CHAIRMAN SALOMON: Well, yeah, I think in this particular case--it is a really good question you asked. That is why I didn't say anything, but what he's talking about now are just pieces of DNA. These are not viruses.

MS. MEYERS: There's no danger of them escaping?

CHAIRMAN SALOMON: Well, this particular instance, yes, there's no danger, but the whole point that we are here, however, is on point for your question, and that is we could generate viruses that, through recombinations of various sorts, did generate new sorts of viruses. So, your question is excellent. I think it threw the speaker off a little bit because his DNA fragments are not viruses.

One of the things you would use this for, though, is a vaccine, so did you do any studies in which you actually used this preparation contaminated with these replication intermediates and injected in a mouse model, let's say, and see whether or not this protein was expressed in the target cells?

MR. LEVY: When we identified that this contained regulatory sequence and that there was a durable percentage of our vaccine preparation that contained this sequence, and, of course, then that would mean if you were to inject this into a recipient, that they would be receiving these regulatory sequences. There are two thoughts, one, these are just mirror images of regulatory sequences that are

already in the plasmid.

However, we do have a very active and thorough toxicology program, where these vaccines are screened in advance through a number of animal studies, and we're looking at not only just the pharmokinetics and distribution and half-life of the DNA entities, but we are actually looking histopathologically at the response to these DNA vaccines--as well as all of these DNAs, of course, are going through the traditional identity testing and safety testing for endotoxin and sterility--sterility--

CHAIRMAN SALOMON: I guess I was just trying to get at--I mean, Abbey is making the point here that a lot of this is so arcane that people here who are not gene experts are not going to follow the implications here. I was just trying to translate a little bit that, in a medical instance, in use, there are clinical problems here, is one might, through the generation of these sort of anomalous DNA species within a vaccine, now taking your product, you would be, let's say, exposing the individual eventually to expression of proteins that might represent new molecules or neoantigens, than if these were based on sequences, let's say captured from proteins that are normally present, one could generally create--potentially, rather, create an autoimmune disease, accidentally, or some other sort of immune complex disorder that might be totally unexpected,

without knowing that you had done this. I think that is the question that we were asking.

MR. LEVY: Okay. I understand. Do you want me to answer that just globally or in our particular case?

CHAIRMAN SALOMON: No, I don't think this is relevant exactly to your situation, so I'm not worrying about your 600 base pairs, but just in general, that is what Abbey was asking.

MR. LEVY: Well, it is something that--in more fully characterizing what it is that you are actually developing and finding that there are some things that are new or different or unexpected, particular in the case of producing new proteins, if your gene is producing a new open reading frame, certainly. We had an instance where there was a question about open reading frames, and even if it means changing a single amino acid, certainly there are plenty of things out there in the literature that imply that changes of even that can be dramatically immunogenic or have a disease potential.

So, I think you have to bring to bear the knowledge you have in the field, but, at the same time, be willing to actually act on even things that would be seemingly subtle, because the potential for them to actually have dramatic effects in humans is certainly there.

CHAIRMAN SALOMON: Thank you.

DR. CHAMBERLAIN: I would sort of like to make the general comment, though, that in some ways, these are not unique issues that apply only to genetic vaccines, that the possibility of rearrangements or not completely characterized products is an issue that has to be dealt with with any type of vaccine, whether it is an attenuated virus, a completely killed virus or a fusion protein that has been purified. There is always a potential for uncharacterized biological material to be in there and enter the vaccination protocol, so this is an issue that pertains to any type of vaccination. It is not unique to genetic vaccines.

CHAIRMAN SALOMON: Any other questions? Thank you. Then we will move to the discussion period. The one thing I want to clarify during the discussion period here is the speakers have presented information at the request of the FDA that was specific, and it is something that, as chair, I have pushed, that we get specific information presented to the committee, so that we get away from just always dealing in generalizations, because I think this is very critical in sharpening the kind of discussions we have.

However, I want everybody to realize that, in the discussions now that follow, the only thing that is off-limits is going back to these speakers and putting them on the defensive about their individual products, because that was never the intention of today's meeting. If there are

intellectual points that evolve directly from the talks, that is excellent, and go at them, but put them in general terms, because we certainly do not want to bushwhack any of these guys who have done us the courtesy to come and really present their data to us and suddenly mess up, you know, a clinical trial that they are in the middle of evolving from Phase II to Phase III pivotal trials.

That is only thing that is off-limits today, but otherwise it is all there, and forward-thinking statements are perfectly encouraged in the following discussion. I have been asked by Dr. Anderson that, before we get started on the individual questions, that he promises me he has a three-minute general perspective, and I have also told him that we're not going to spend a half-hour discussing it, but anyway, out of respect to Dr. Anderson, I think it is very reasonable and I look forward to his general comments.

DR. ANDERSON: I am here as a gadfly. I will raise an objection or raise concern about every single issue the FDA has brought up, and I want to put that in perspective at the beginning. I have had many, many discussions with the FDA over the years, all of them friendly, usually friendly. This one really is part of what I think is an absolutely invaluable process of having public discussion of issues.

If this were a real world--I take that back--if

this were an ideal world, if we had infinite time, infinite money, that everything that could be done to help patient safety would absolutely be fine, but it is a real world. We deal with real patients. We deal with real budgets.

Therefore, what is critical and what these two days are designed to do is to look at balance, is to balance priorities, and I know this is what the FDA has the meeting to do. In fact, that is the reason I was asked to come, because I will provide a counterbalance perhaps to other views. If one plots, and everybody in this room knows plots, patients safety versus cost, what everyone knows is initially there are many things that can be done at minimal cost that really benefits patient safety, and as one goes up in terms of cost, patient safety starts to fall over a bit, and then it becomes asymptotic.

You can pour in tons of money and the increase in patient safety becomes really very minimal. The issue is where are we in that curve for each individual recommendation, and many which I might object to, I actually agree with, but that--my role here is going to constantly look where are we on that curve. I believe, as the FDA knows, that the increased monitoring requirements is right on that steep slope, will make better protocols, and that is well worth the extra money.

Well, is there any reason why one should not say,

let's not worry about the money, let's go all-out, because anything that improves patient safety is a good thing? Well, no, everything has repercussions, and the repercussions in this case could be catastrophic, and that is what I want to point out. First, let me tell you where I'm coming from. I have been involved in 16 clinical protocols. I'm involved in two now that are at the pre-IND/IND stage. Of those 18 clinical protocols, the two present ones involve no commercial money at all. It is all gift money or grant money.

Of the other 16, most involved either a start-up, a small or a medium-size biotech company, all of it GTI, one of it was a large pivotal Phase III, done by big pharma, Novartis, and I was a consultant to that, the brain tumor trial. So, I have firsthand experience in all aspects of how you do clinical trials and who pays for them, and the thinking behind what goes into doing them.

Having said that, what are the repercussions of going up in this curve when you can superimpose another curve on this curve, and that is what happens to the initiation of clinical trials? Increased cost is not going to affect big pharma much at all. It is not going to affect major biotech much at all, but it is going to affect physician-sponsored INDs where the budget is really very minimal. It is going to affect new, small biotech

companies. That is where the effect is going to be.

Many of the most innovative protocols are going to come from physician-sponsored INDs and small companies. So, the more you inhibit those trials from taking place, the more damage you are going to do to the field as a whole. Cancer research isn't going to be particularly affected. Cardiovascular disease is not going to be particularly affected, but rare genetic diseases, a cause that Abbey and I have been fighting for for decades, I guess, is going to be catastrophically affected.

If one superimposes on this plot, here is your patient safety versus cost, in terms of physician-sponsored INDs, it is going to go high and then gradually drop and then precipitously drop as the cost goes up. What I will argue on every point is where are we on the curve? Is the amount of increased patient safety balanced by the loss of physician-sponsored INDs? That is where I'm going to be coming from the entire time. Do you feel comfortable now, Mr. Chairman? You were really worried, turning the microphone over to me.

CHAIRMAN SALOMON: I am comfortable. I think that the issue here that has been framed by Dr. Anderson is really pretty much the charge that I believe we have as a committee, and that is to see two sides, actually, if not three sides of this area. One is that we have a

responsibility to the public. Henceforth, we have public meetings and invite the public to participate in these sorts of discussions.

The second is we have a responsibility to the field in the sense that we are trying to deal with new areas. Obviously, if these were well-established areas, they don't need advisory committees for it. As we deliberate on these things, I also agree with Dr. Anderson that we need to be cautious, that we do not make recommendations in regulatory fervor that would damage the ability to progress in this field, because in a way that is also our agreement with the public, that science is going to move forward, but that science is going to move forward responsibly.

I think the last thing that really is particularly important here, and I think it is going to be how we respond to Dr. Anderson's point, and that is this positive tension between the development of things in academic centers, by individual investigators and small pioneer companies, and the transition to large trials and big pharma, in which case you're dealing with many patients and probably the only thing I would probably take issue with, Dr. Anderson, is that we need to think of that as two separate issues. I mean, there may be points at which we demand less in order to facilitate the first transitions of novel technologies,

but then later crack down and accept the higher expense as the public is exposed more to these things. Yes?

DR. GORDON: I just wanted to speak to something related to that, although you said you didn't want to belabor these points for half-an-hour, so I'll keep it under half-an-hour. It would seem to me that what we're seeking here is the best possible testing paradigms for quality of preparations and characterizing preparations.

What one wants then is to find those and then to implement them with uniformity for all preparations that are going to be administered to people. Some of these testing paradigms may prove to be costly and involved, and because they are costly and involved, their uniformity is much less likely to be maintained, if it is done in 1,000 different places over the country by 1,000 different groups, who have 1,000 slightly different ways of doing this or that technique.

Overall, when one looks at this problem, one has to think of methods of establishing uniformity and reproduceability for these tests, and deciding whether or not that is something which should be left to individual entities to do anymore, or whether or not we should consider referral centers for quality-control testing.

CHAIRMAN SALOMON: Good point.

DR. MILLER: I just have one clarification, when

we discuss these questions for session one, is there a distinction between the in vivo and ex vivo use of vectors, and when and how can we determine if there is any distinction, from a safety standpoint and from what has already been--cell lines that have been set up years ago with vectors, and are we going to then subject them to the same scrutiny as what is going in now into patients? I just wanted some clarification on that.

DR. WILSON: We don't believe that the discussion should be delineated along those lines, of ex vivo versus in vivo. The question you raise about those trials that have established allogeneic cell lines that were transduced ex vivo some number of years ago, and how we would apply any new recommendations coming from today's discussion is an important one, but I think that for the purposes of these questions I would rather just lump them both together.

CHAIRMAN SALOMON: Last comment, Dr. O'Fallon.

DR. O'FALLON: This will be very brief. I have a profession only because there is variability in systems, and we struggle in all of our contexts to separate out that variability that we can explain and hopefully then control from that variability which, until we understand it, we call randomness. We are exposed to some questions here in the next few minutes we're going to discuss that is essentially going to be talking about controlling variability in a

system which I maintain still is going to have massive amounts of random variability connected to it, so these issues have to be balanced as we are discussing this.

We do not want to see how many things we can put on the head of a pin until we decide how many pins we have.

CHAIRMAN SALOMON: That is probably an excellent introduction to the next part. Let's get started. The one thing I might suggest to everybody, it is very useful to me, is in the material that was put together by the FDA, there is background information titled, "Structural Characterization of Gene Transfer Vectors," and at the very last page is a table that gives you essentially all the common vector classes, which, I think, for everyone who is not instantly conversant with the different sizes of the genomes of these different vectors and some of the replication properties, et cetera, you just might want to have that in front of you. It's just a suggestion.

Question one is for vectors up to 40 KB in size, which, referring to Table 1, would include plasmid, retroviral vectors, and adenoviral vectors and adeno-associated viral vectors, but would specifically exclude herpesvirus vectors, poxvirus vectors and some other EBV virus vectors, which is a type of herpes vector, et cetera, so there is a delineation here, is the point I'm making.

For vectors up to 40 KB in size, the FDA proposes

that the full sequence, coding and non-coding, should be determined prior to initiation of a Phase I clinical trial. Do you agree with this proposal?

DR. GORDON: It sounds good, but let me just say that I think there are some problems with it, political mainly. I mean, I think it will be very difficult to explain to somebody receiving a larger vector why theirs wasn't sequenced, and if you are establishing sequencing as the gold standard, you're going to put yourself in the position of deliberately applying less than the gold standard to some individuals and not to others.

CHAIRMAN SALOMON: Yes, I actually agree with that. Let's divide that question into two, because we will get that in the next question. It is absolutely on point, however. The first question I'm trying to keep relatively simple, and that is, for a vector under 40 KB, would you agree that the full sequence should be provided to the FDA at the time of the initiation of a Phase I investigator-initiated or other trial?

Dr. Anderson?

DR. ANDERSON: The issue here is Phase I, because you're certainly going to do it higher up, and anybody who would go into a $100 million clinical trial and not have that information, just it is not going to happen. We are only talking about a Phase I, and the problem with doing

this or anything else is that it adds an additional increment. If the NIH is willing to fund it, well, that is fine. I mean, every one of us would love to send all of our material and have it all sequenced for us. That is great, but it costs money. What are you going to do with that information? If it turns out that you have a vector and it has got some salmon sperm DNA, and it's the salmon sperm DNA that cures cystic fibrosis, well, wonderful. What difference does it make? At a Phase I trial now, where you're only talking about a few patients and you're looking at safety and you're looking for a little bit of efficacy, as the FDA is prone to say, if it works, that's what is important, so long as it doesn't hurt the patient and it works.

To require physician-sponsored INDs to sequence everything up front, before they ever go forward, might be a good idea. Nowadays, it is pretty cheap to do. But it is going to be the same fundamental issue every time you require an additional step and an additional requirement that really doesn't have a real need in a Phase I trial, you're going to reduce the number of trials.

CHAIRMAN SALOMON: Dr. Mulligan?

DR. MULLIGAN: I mean, I can't disagree more. I think this is the most no-brainer of a question. This is the standard way people do science at this point, and the

availability is such that the cost is negligible. When we get to point two, I'm going to say we ought to do that, too, but with regard to this question, I think it would be tremendously irresponsible to conduct any clinical experiment without having a DNA sequence.

Our own personal experience at a major Ivy League university, in receiving DNA samples from people to help them make vectors, is that close to 50 percent of the things you get in-house by very reputable people are not as advertised. I hope we don't spend more than about five minutes on this question.

CHAIRMAN SALOMON: Well, this is public discussion.

DR. SAUSVILLE: Yes, I would heartily agree with Dr. Mulligan on this point, and where I respectively disagree a little bit with Dr. Anderson is that Phase I is safety and that is the linchpin of what you are going to build subsequent decisions on, and you could potentially make a bad decision by not knowing exactly what you have. I would agree with the characterization of Dr. Mulligan and strongly call for that.

DR. TORBETT: I will have to agree with Dr. Mulligan; when you start off these kinds of procedures, I think it is first knowing what you have in hand and going forward is the critical first step. This is the beginning

of the whole process, and I think knowing the sequence and having the same experience as Dr. Mulligan, we do sequence most of our vectors.

We get them from others, because there are number of errors. However, that being said, when unknown sequences are found, I think going to a standard database is useful as long as that sequence is present in the standard database. That is, I think, something we have to consider, that is, unknown sequences, I think, need to be documented in different kinds of vectors.

CHAIRMAN SALOMON: I guess the point I would like to add is the research we're doing should be hypothesis-driven. I don't need to tell Dr. Anderson that. He helped teach me that. One of the things that would be a true event is that if salmon sperm DNA cures cystic fibrosis. I mean, if it does, I would be very happy for cystic fibrosis, but I think we have to have sequence, too, so I happen to agree with that.

Dr. Anderson?

DR. ANDERSON: Having made my gadfly point, every vector we have, we sequence totally. We sequence it repeatedly through the entire process. It is absolutely required, and Richard, the only thing I disagree with, and on our West Coast, we don't get 50 percent of them right. Most of the things we get in are about 80 percent are wrong.

Our rule of thumb is if you get it from your best friend in the next lab, you sequence it.

CHAIRMAN SALOMON: One more comment.

DR. GORDON: I just want to say there may be a way of harmonizing these two very disparate points of view, and that is the difference between having sequence in hand or making sure that sequence is becoming available as one moves forward. The reason I say that is because I think it would be very unusual indeed--suppose salmon DNA was found in a vector, would that then cause you to discard the vector or not? What are we going to do with the information, is basically what I'm saying.

I think most of the time we're not likely to do much with it except shrug our shoulders and say, "Gee, that is interesting," and then go forward. I think one way to harmonize this issue with issues of dauntingly difficult sequencing is to say that samples of this material have to be made available for sequence ongoing and introduction into some sort of meaningful database.

CHAIRMAN SALOMON: The point that I am not so comfortable with is that if you get sequence and if there is anomalous sequence present, that you just say, "Well, you know, it is present. I'm going on to the clinical trial," I'm not sure I'd buy that actually. One of the things that we will get to in question five is what to do when you find

anomalous sequence, you know, what would be a limit--I think right now I would like to just deal with the question should we have sequence, and I think we're getting pretty close to agreeing that we should have sequence, and you raise now the additional and important point of what you do when the sequence doesn't match, and I think we can't leave today without having addressed that issue.

DR. SAUSVILLE: You may want to discuss this later, but I would certainly say it depends on the stage in which you discover it. If it is the beginning of the process, I mean, this is technology, this can be fixed.

DR. O'FALLON: So why is nobody asking whether it should be 40 or 35 kilobytes or 45 kilobytes or--I mean, how did we arrive at that? And that is where I assumed we would see some of this discussion. I don't really think Dr. Anderson was voting against this.

DR. ANDERSON: No, the reason it is 40 is that encompasses the adenovirus, the retrovirus and the plasmids. That is why nobody has objected to 40.

DR. WILSON: I think also the other reason we cut it off at 40 was to take into account some the comments Dr. Anderson was reflecting earlier, in terms of the cost versus the benefit, so when you go into the larger classes, which is question two, where you're looking at a couple of hundred kilobases, the cost becomes much more of an issue.

CHAIRMAN SALOMON: Carolyn, can you, just for the committee's sake and the public, tell us what you guys found? You did some research on what it would cost at a contract lab to do 40 KB of sequence.

DR. WILSON: At a contract lab, which is probably, for most people, the most expensive way to do sequencing, most people would have access to in-house automated DNA sequencers, it would cost around $20,000 to $22,000 to do a typical adenovirus vector.

CHAIRMAN SALOMON: Would that be both strands of a double-strand DNA? Total? Not $44,000? About $22,000, and we could probably do that for half at an academic center or at the NIH.

DR. O'FALLON: It seems to me then, let's assume it's past and I said this to Jay at break. I hope that wasn't illegal, but this will become obsolete, because that $22,000 will be going down to $10,000, will be going down to $200, in which case we can move this up to 100. I like the other answer better, which is saying that it encompasses most of the current viruses we're interested in. It will certainly cost less, even before any recommendations can be published.

CHAIRMAN SALOMON: Phil, did you want to make a comment?

DR. NOGUCHI: That's a valid point. I think some

of the further discussion will also illustrate that this is what can be done with an actual virus without further manipulation. To get to the larger viruses, you will probably have to re-manipulate the virus itself or the vector. That introduces then again another error of randomness to it or lack of scientific understanding that makes that problematic. So, 40 KB is what is practical, and what can give us accurate information, as well, I think, at the present time. Maybe next week, it will change.

CHAIRMAN SALOMON: I have been asked to call for a vote on this particular question, specifically then that we would agree to recommend to the FDA--

DR. SIEGEL: Given concerns about hand-counts, we've prepared some punch cards.

CHAIRMAN SALOMON: Only if representatives from industry, pharma, university and the NIH are all present.

DR. WILSON: Right. We're going to distribute some punch cards. Is that not it?

CHAIRMAN SALOMON: Very funny.

DR. GORDON: Just a quick procedural question. I'm sort of an ad hoc or a visiting member and I was under the impression, when recruited, that I was a nonvoting member, but in the preamble this morning, the implication was that I would be voting. Please instruct me.

MS. DAPOLITO: Yes, you have been appointed as a

voting member. I apologize if that was not made clear. You do have the option of abstaining.

CHAIRMAN SALOMON: Again, for those of you have not been on the committee before, I just want to clarify that these votes are not going to determine the next President or the next FDA policy, for that matter. These are recommendations of the committee and they're meant to just give a very clear message on specific points. We won't vote on everything in the next two days.

With that said, I think everybody is clear about what the motion is, and a set of hands would be okay, Jay? Those who say aye, if they could please raise their hand.

[Show of hands.]

MS. DAPOLITO: 11, yes.

CHAIRMAN SALOMON: And nays?

MS. DAPOLITO: One nay, so we are one shy.

CHAIRMAN SALOMON: Okay. Actually, in the interests of making sure we have discussed this whole thing, I'm not sure we came up with a reason why you would vote nay, so could you explain that, just because again my job, I think, is to make sure that all the sides of this is on the table.

DR. GORDON: If this is causing some sort of grinding halt to the proceedings, I can abstain or something. I felt the discussion did not result in an