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The human *LINE-1* retrotransposon (*L1Hs*) is known to cause mutations by inserting into genes and inactivating them. The element is expressed in many breast tumors and breast tumor cell lines, suggesting that *L1Hs*-induced mutations may play some role in this malignancy. To test this hypothesis we plan to place a "tagged" *L1Hs* element in non-malignant breast epithelial cells and let it transpose. If the cells become malignant, we can isolate the gene(s) into which the element has transposed by using the unique tag. Previously we tested a number of plasmid and retroviral vectors in several cell lines without success. We have recently found that a non-integrating "tagged" *L1Hs* vector may be useful if we can succeed in increasing the number of retrotransposition events/transfected cell population. In addition, we are using the expression vector pIRES1neo (producing a bicistronic mRNA) to express *L1Hs*-encoded proteins in transfected MCF10A cells. This system will be useful to test the effects of expression/overexpression of *L1Hs*-encoded proteins in non-malignant cells.

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FOREWORD

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Tom Fanning Oct. 25, 1997

PI - Signature Date

Table of Contents

Introduction

Human *LINE-1* (*L1Hs*) is a transposable element that encodes a reverse transcriptase and moves *via* an RNA intermediate [1]. It therefore seems possible that cells in which *L1Hs* is active may be subject to insertional mutagenesis. We have recently found that the element is expressed in a significant number of germ cell cancers [2,3], and in many breast tumors and breast tumor cell lines [4,5]. These findings raise the possibility that the initiation or progression of malignancy in certain steroid hormone responsive tissues is facilitated by *L1Hs* expression and/or transposition.

In addition to insertional mutagenesis, there are several other characteristics of the *L1Hs* element that suggest its potential as an oncogenic agent. For example, *L1Hs* has an internal promoter which could potentially lead to readthrough transcription and activation of downstream genes. In addition, the *L1Hs*-encoded p40 protein has a leucine zipper motif, suggesting a possible interaction with other cellular proteins. Such interactions at inappropriate times might lead to the disruption of important cellular functions. Thus, *L1Hs* involvement in cancer could occur by several mechanisms, either singly or in combination.

Our long range goals focus on the isolation of cellular genes that are affected by *L1Hs* transposition. These genes are presumably the ones whose inactivation (by insertional mutagenesis), or activation (by readthrough transcription), is one of the steps in the pathway leading to malignancy. Specifically we have proposed to:

(1) Place a "tagged", transpositionally competent *L1Hs* element into non-malignant human breast epithelial cells.

(2) Identify malignant cells arising from the non-malignant cell population and isolate and characterize sequences into which the tagged *L1Hs* element has transposed.

Experimental

(a) Transfection of breast epithelial cells.

We have used three human, non-malignant breast epithelial lines in our work: HBL100, Hs587Bst, and MCF10A. Transfection protocols have utilized; (a) calcium phosphate-DNA co-precipitation, (b) a DEAE-dextran-DNA mix, and (c) several liposome-DNA complexes (LipofectAMINE, LipofectACE, and Lipofectin, all sold by Gibco-BRL). Our initial experiments utilized a β-galactosidase-containing vector, since measurement of the enzyme activity in cell extracts is very sensitive. Using this vector, both the HBL100 and Hs587Bst cells were completely refractory to transfection. Control transfections utilizing COS cells were successful (COS is an SV40-transformed monkey kidney cell line that is easily and efficiently transfected). Thus, we have concentrated our efforts on the MCF10A cell line.

Transfection of MCF10A cells with the β-galactosidase-containing vector suggested that transfection was possible, but the efficiency was low. Indeed, when we stained a population of transfected cells, it was apparent that less than 1% were actually taking-up DNA under our most optimal conditions. Nevertheless, this is certainly sufficient to give us stably transfected cells that express *L1Hs*, which is our goal. To obtain stably transfected cells we treat with a LipofectACE-DNA complex, then subject the cells, after an appropriate time interval, to a neomycin analog (G418) since the vector contains both the *L1Hs* p40 gene and a neomycin (neo) resistance gene. Neo resistant cells are cloned, grown in large numbers, and assayed for p40 expression.

(b) Expression of the *L1Hs* p40 gene in transfected cells.

In our initial experiments we have used vectors containing only the first *L1Hs* open reading frame (p40 gene). We have done this for several reasons; (1) we have an anti-p40 antibody that is capable of detecting small quantities of the protein. Thus, we can easily assay cells for *L1Hs* expression. (2) Experience gained in this work will be very useful in placing the full-length *L1Hs* element into the same cells.

We have placed the following plasmid-based constructs into the MCF10A cell line: RSV-p40, CMV-p40 and MMTV-p40. These vectors place the *L1Hs* p40 gene behind the Rous sarcoma virus promoter/enhancer, the cytomegalovirus promoter/enhancer and the mouse mammary tumor virus promoter/enhancer, respectively. All constructs proved to be capable of expressing the p40 protein, as shown by Western blotting extracts from transfected COS cells. In addition, we have placed the p40 protein into several retroviral vectors: LXSN, LNSX and Elneo [6,7]. The LXSN and LNSX vectors have two promoters, one for the neo gene and one for the p40 gene. The Elneo vector contains only a single promoter/enhancer. With this vector the neo and p40 genes can both be expressed due to mRNA splicing. Thus, complete transcripts express the p40 protein which is located at the 5' end of the mRNA, while spliced mRNAs express the neo gene product. All of the tested constructs produced stable, drug resistant colonies after transfection into MCF10A cells. However, in no case was p40 detected.

We assume, but have not yet shown, that for vectors with two promoters the promoter and/or enhancer driving the expression of the p40 gene is inactivated, probably by methylation. The promoter/enhancer for the neo gene, which is under strong selection, is obviously still functional. Exactly why the Elneo construct produces no p40 is unknown. We have been informed by colleagues that in some cells virtually all mRNAs from this vector are spliced. Apparently MCF10A is such a line.

(c) The bicistronic expression vector pIRES1neo.

Clonetech Laboratories, Inc. of Palo Alto, CA, introduced a new expression vector, pIRES1neo, in late 1996. This vector was constructed because many laboratories have experienced difficulties, similar to ours, expressing certain genes in some cell types. The vector has the following properties: transcription is driven by a CMV immediate-early promoter/enhancer. Following the CMV region is a multiple cloning site and then an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus. Following the IRES region is a neo resistance gene. The transcript produced by the construct contains the gene of interest and the neo gene on one mRNA, and both can be translated due to separate ribosome binding sites. Thus, G418 resistant cells should, barring deletion, also express the gene of interest.

We have currently cloned both *L1Hs*-encoded proteins into this construct, and transfected them into MCF10A cells. A low level of expression occurs from the uninduced CMV promoter. We are able to increase the amount of expression considerably by inducing the promoter with small amounts (10 nM) of the tumor-promoting phorbol ester, TPA [8]. There does not appear to be a loss in inducibility over time: we have a transfected cell line, constructed over 6 months ago, that still expresses large amounts of p40 when treated with TPA. We are currently using this line to examine the effect of p40 overexpression on the cell, but have no definitive results yet. In addition, we are investigating other agents that might be more suitable than phorbol esters in inducing p40 expression since there is a good deal of "cross talk" among protein kinase cascade pathways[9].

(d) The JM101 vector.

We have recently obtained a "tagged" *L1Hs* element in the extrachromosomal expression vector, pCEP4. This construct, JM101, was made available by colleagues studying the *L1Hs* retrotransposition process [11]. To date we have transfected this vector into MCF10A cells to investigate the rate and extent of retrotransposition in this cell line. It had been reported that there are about 750 retrotransposition events per million vector-containing HeLa cells [11]. In the experiments we have done we find many fewer than that with MCF10A cells. This is probably not a function of transfection efficiency since, following transfection, vector-containing cells are selected with hygromycin. Then, after a week or more, the cells are challenged with neomycin to select those in which transposition events have occurred.

In order to boost the number of retrotransposition events in MCF10A cells we have treated the JM101-containing cells with a small amount of TPA after 3-4 weeks of hygromycin selection. However, no increase in retrotransposition events (neo resistant cells) were seen. This suggests to us that after transfection of the construct into MCF10A cells there is a burst of transcription and retrotransposition. After the cells have been grown for 3-4 weeks it is difficult to induce transcription from the CMV promoter. It is possible that the CMV promoter is slowly being shut down, although the hygromycin promoter is obviously functional. Whatever the cause, we are now trying to induce transcription by TPA treatment several days after transfection of the vector into the MCF10A cells. We hope that this will result in a large number of *L1Hs* transcripts and a corresponding increase in the number of retrotransposition events.

In addition to the work with JM101, we are preparing stable cell lines expressing the complete *L1Hs* element from the bicistronic pIRES1neo vector. These lines may enable us to more easily manipulate the total amounts of *L1Hs* transcripts in the cell by manipulating the promoter of a stably integrated gene.

Conclusions

A successful execution of the goals of the grant require that a full-length, transpositionally competent *L1Hs* element be placed into a non-malignant breast epithelial cell. After a number of failures, we have two vectors (pJM101 and pIRES1neo) that may be suitable for our purposes. The first vector contains a "tagged" *L1Hs* element that is known to be capable of retrotransposition. We are currently trying to increase the total number of transposition events with this vector to a satisfactory level. The second vector produces a bicistronic mRNA and allows the overexpression of cloned genes by transient activation with phorbol esters and possibly other agents. Thus, Aim 1 of the grant (vector construction and expression in non-malignant breast cells) will be accomplished soon. Then, we can concentrate on isolating any malignant cells that might arise and identifying the genes involved.

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