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Comparative Analysis of Artificial Antisense RNA Regulation in Fission Yeast and Human Cells

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The fission yeast *Schizosaccharomyces pombe* has recently been established as an experimental model for the study of antisense RNA-mediated gene suppression. To validate the use of *S. pombe* as a host for identifying antisense genes for use in human cells, it was important to determine if sequences identified in yeast were as equally effective in a human cell line. This report describes the comparison of a range of *lacZ* antisense RNAs targeting a *lacZ* gene expressed in HeLa cells in a comparable manner to its expression in *S. pombe* cells in earlier studies. In both cell types, the same *lacZ* gene target was expressed using the same promoter. Antisense genes were expressed episomally in both experimental systems and the levels of suppression determined. In all cases, the relative level of suppression of the *lacZ* gene was similar in the mammalian and yeast cells. This result indicates that, at least for *lacZ* antisense RNA, results obtained in fission yeast are predictive of their behavior in the mammalian cellular environment.

Key Words: **antisense RNA; HeLa; lacZ; fission yeast.**

The intrinsic ability of complementary nucleic acids to hybridise has opened the way for the design of sequences that have the potential to interfere with the expression of genes in a specific manner. As such, the use of antisense RNA to regulate gene expression is well established as a basis for the development of gene therapeutics and as a tool for elucidating gene function. Inhibition of target genes is thought to involve one of a number of mechanisms including interference with pre-mRNA splicing, prevention of mRNA transport, inhibition of ribosome assembly and induction of dsRNA-specific nucleases (1). Although there are many examples of successful antisense RNA-mediated gene suppression, it is still unclear which mechanism predominates and how an RNA molecule can be used to optimally harness its potential. In order to address these fundamental questions several groups have attempted to characterise parameters of antisense function in a range of mammalian cell lines (2). However, the use of mammalian cell systems is expensive and time-consuming and is therefore more labour intensive than the use of traditional model eukaryotes. As a result, several groups have sought to develop yeast species as hosts for the study of mechanisms of gene suppression (for reviews see references 3, 4).

Arndt *et al.* (5) described a strain of the fission yeast *S. pombe* that stably expressed the bacterial *lacZ* gene from a chromosomal position. Expression of a range of antisense RNAs within the same cell resulted in partial suppression of the target *lacZ* gene. This result established the fission yeast as an ideal model in which to study antisense function and to identify antisense RNAs effective against non-yeast target genes. However, it remained to be determined how valid this species was as a model for the study of antisense RNA intended for application in human cells. An important experiment therefore, was the determination of whether the antisense-mediated suppression observed in yeast cells was similar when the same *lacZ* antisense constructs were tested in a mammalian-based assay system against the same target gene.

This report describes the comparison of a range of *lacZ* antisense RNAs targeting a *lacZ* gene expressed in HeLa cells in a similar manner to its expression in *S. pombe* cells described in earlier studies (5). We show that the efficacy of antisense RNA in fission yeast cells is comparable to that seen in HeLa cells and we suggest that this result validates *S. pombe* as a model for the development of strategies to suppress target genes in human cells.

EXPERIMENTAL PROCEDURES

Yeast strains, media, transformation. *S. pombe* strain KC4-6 has an SV40 early promoter-driven *lacZ* gene integrated at the *ura*4 locus as described by Arndt *et al.* (5). All yeast strains were maintained on standard YES or EMM media and were manipulated

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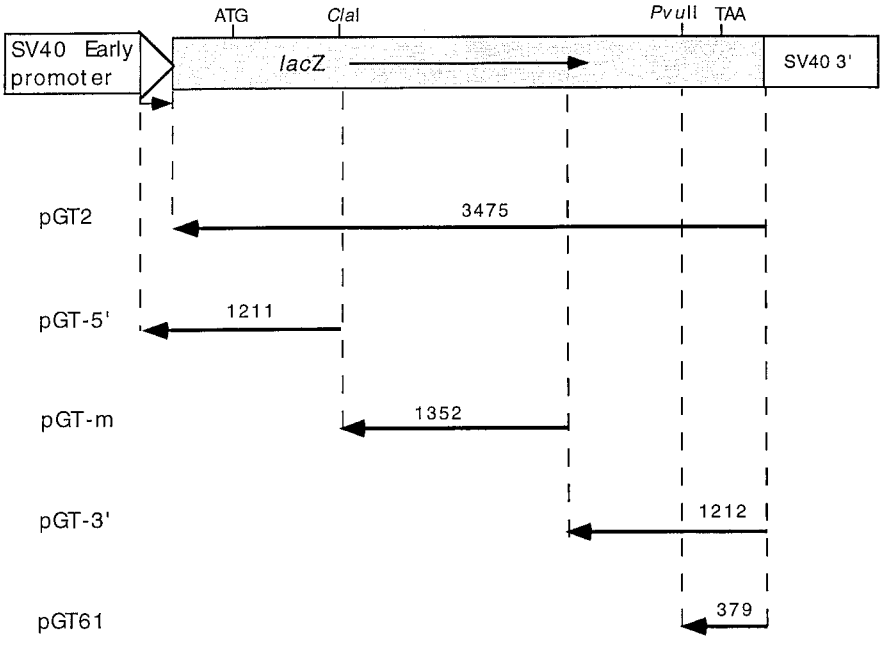

FIG. 1. Schematic representation of the *lacZ* target gene and antisense sequences. The *Escherichia coli lacZ* gene, controlled by the SV40 early promoter and 3′ processing signal, is shown (top). In *S. pombe* the *lacZ* gene is integrated at the *ura4* locus on chromosome III. In HeLa cells the *lacZ* gene is located on the pSVβgfp plasmid. The start site and the direction of transcription from the SV40 promoter at position −298 is indicated by the bent arrow and the *lacZ* open reading frame is shown to extend from the ATG (+1) to TAA (+3141). Arrows indicate the direction of transcription of the target *lacZ* gene (top) and the antisense DNA sequences transcribed from the various pHOOK plasmids (directly below). There is a 23-bp overlap between pGT-m and pGT-3′.

according to Moreno *et al.* (6). Yeast cells were transformed with plasmid DNA by electroporation (7).

Gene construction for expression in S. pombe cells. All routine DNA manipulations were completed using standard protocols (8). The construction of the *S. pombe* plasmids encoding the long (pGT2), 5′ (pGT-5′ previously designated pGT59), and short 3′ (pGT61) *lacZ* antisense genes and their corresponding sense control plasmids have been described previously (5). Additional plasmids encoding the mid-region (pGT-m) and long 3′ (pGT-3′) *lacZ* antisense genes were also prepared. PCR fragments were generated from the template pNEBβ2 plasmid DNA (5) using the following primers: mid-region 5′-AAGAGATCTAGCGTGGTGGTTATGCCGATCGCGTC-3′ and 5′-GGAAAACCTCAGTGTGACGCTCCCCG-3′; long 3′ antisense primers were 5′-AAGAGATCTAAAACCTCAGTGTGACGCTCCCC-GCC-3′ and 5′ AAGAGATCTCAATGTATCTTATCATGTCTGGA-TCC-3′. In each case a *Bgl*II restriction site was added to both the 5′ and 3′ ends of the amplified DNA fragments. The mid-region spanned positions +913 to +2265, while the long 3′ was located between +2242 and +3454. These positions are based on the pSVβ sequence map (Clontech) and +1 corresponds to the ATG. Fragments generated by PCR were cloned into the *Bgl*II site in pSP72 (Promega) and subcloned as *Bgl*II fragments into the *Bam*HI site of pREP1 in the antisense orientation. All antisense RNAs were expressed under the control of the *S. pombe nmt*1 promoter from the plasmid pREP1 containing the *S. cerevisiae* LEU2 selectable marker (9). The target *lacZ* gene and all antisense fragments tested are shown schematically in Fig. 1.

Gene construction for expression in HeLa cells. The *lacZ* target gene used in HeLa cells was expressed from the pSVβ plasmid (Clontech) and was modified by the insertion of a fragment encoding the CMV promoter, *gfp* cDNA encoding for green fluorescent protein and the SV40 polyadenylation signal. The *gfp* cDNA was isolated from pGREEN LANTERN (Gibco-BRL) by PCR amplification using primers 5′-TTCGCAAGCTTCGATCCAGACATGATAAG-3′ and 5′-CTAGGATGCATAAACAGCATCGTGCAGGTC-3′ and cloned as a *Hind*III/*Pst*I fragment into pSVβ to produce the pSVβgfp plasmid. Antisense RNA-encoding DNA fragments studied in yeast cells were directly subcloned from the pSP72 yeast expression vector into unique *Bgl*II or *Bam*HI sites of the mammalian expression vector, pHOOK2 (Invitrogen).

Cell culture, transfection, and flow cytometry. HeLa cells (ATCC CCL-2) were maintained in Dulbecco's Modified Eagle's Medium containing 10% bovine serum and penicillin (100 U/mL)/streptomycin (100 μg/mL) and were incubated at 37°C and 5% CO_2. Subconfluent HeLa cells were transfected following harvesting by trypsinization. Cells were resuspended at 1×10^7 cells/mL in phosphate-buffered saline (PBS) and 500 μL aliquots were used for each 0.4 cm electroporation cuvette containing 20 μg DNA (20 μL) followed by pulsing at 960 μF, 400 V using a Gene Pulser with a capacitance extender (Bio-Rad). Cells were transferred immediately to medium, pre-equilibrated to 37°C and 5% CO_2 and harvested at 41–44 h by trypsinization and washing with PBS. Cell pellets were snap-frozen in liquid nitrogen and stored at −70°C before analysis. For co-transfection studies 2 μg of the target plasmid and 18 μg of the antisense or sense pHOOK2 plasmid were mixed thoroughly before addition to the cuvette. Fluorescent-activated cell sorting (Becton Dickinson FACSORT) for *gfp* expression was carried out on HeLa cells that were harvested by trypsinization and washed with PBS.

β-galactosidase assays. β-Galactosidase enzyme activity was determined in fission yeast using a cell permeabilization protocol as previously described (10). HeLa cells were assayed using the β-Gal ELISA kit (Boehringer Mannheim) according to the manufacturer's instructions.

Analysis of RNA. All yeast RNA manipulations were as described in Arndt *et al.* (5). RNA was extracted from HeLa cells (stored at −70°C) using the Total RNA isolation reagent (Advanced Biotech-

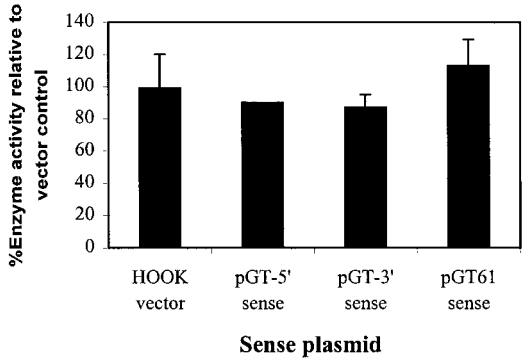

FIG. 2. Co-transfection of HeLa cells with sense control plasmids and the target pSVβgfp. β-Galactosidase activity is expressed as a percentage of the empty vector control. Each bar of the histogram represents the average of three independent co-transfections. Each transfection was then assayed in triplicate for β-galactosidase activity. Standard deviations are indicated by the error bars.

nologies Ltd) according to the manufacturer's recommendations. A PolyATract mRNA isolation system (Promega) was then used to isolate mRNA from 100 μg aliquots of total RNA. Northern analysis was performed using standard techniques (Sambrook *et al.*, 1989) and Hybond N membrane (Amersham). Probes were radiolabelled ([α-^{32}P]dCTP, Bresatec) using the Megaprime random labelling kit (Amersham). Two probes were used, glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Clontech) for the detection of an endogenous transcript and the *Hind*III/*Xcm*I fragment isolated from the pHOOK2 vector. This latter fragment hybridizes to the 3′ polyadenylation signal of all antisense and neomycin transcripts produced from the recombinant pHOOK2 plasmids. Hybridization was carried out using ExpressHyb (Clontech) solution as per the manufacturer's instructions. A phosphorimager (Molecular Dynamics) was used for signal detection.

RESULTS

Expression of the target lacZ gene. The bacterial *lacZ* gene, controlled by the SV40 early promoter and SV40 polyadenylation signals was derived from the pSVβ plasmid and used as a target in both the yeast and HeLa cells. In *S. pombe,* a single copy of the target gene was integrated into the genome using *ura*4 flanking DNA sequences and homologous recombination at the wild-type *ura*4 gene locus (5).

The *lacZ* gene was introduced on the plasmid pSVβgfp into HeLa cells by transient transfection. In recognition of the varying levels of transfection that can be obtained, the *gfp* gene expressing green fluorescent protein was inserted into the target pSVβ plasmid to enable FACS measurement and subsequent normalization of transfection efficiency. The expression characteristics of the *lacZ* and *gfp* genes encoded on pSVβgfp were examined at 20 h, 48 h and 68 h post-transfection. The activity of the *gfp* gene reached a maximum level at 20 h that was maintained until 48 h after which the level reduced by approximately 50% at 68 h post-transfection. Analysis of the expression of the *lacZ* gene post-transfection showed that β-galactosidase activity reached a maximum level at 20 h and then declined in a linear manner until the latest measured point.

In HeLa cells the empty vector control pHOOK plasmid and pHOOK containing the test fragments in the sense orientation were co-transfected with the target plasmid expressing both gfp and β-galactosidase to determine whether there was any differential effect on expression of these two proteins. When plotted, there was a high correlation ($r^2 = 0.88$) between the expression of both proteins from the target plasmid and no significant suppression was observed (Fig. 2). Therefore to simplify the protocol, all antisense results were expressed relative to the empty vector control.

Expression of antisense RNA. The behaviour of three antisense RNA fragments targeting the full coding sequence, 5′ and short 3′ regions of the *lacZ* gene (pGT2, pGT-5′ and pGT61) has already been described in fission yeast (5). Suppression levels were reported as 45%, 20% and 10% respectively. Two additional antisense RNA fragments similar in size to pGT-5′ corresponding to the mid-region (pGT-m) and a longer 3′ region (pGT-3′) of the *lacZ* mRNA were constructed (Fig. 1). pGT-5′, pGT-m and pGT-3′ each covered consecutive, approximately 1.2–1.35 kb regions of the target gene and northern analysis showed that they were expressed in yeast at similar levels (Fig. 3).

The same antisense RNA sequences used in fission yeast were directly subcloned from the yeast expression vector into the mammalian expression vector, pHOOK2. Northern analysis of HeLa cells transiently transfected with pGT2 showed mRNA levels with a peak at 20 h, followed by an 80% reduction by 48 h. Expression of pGT-5′, pGT-m, pGT-3′ and pGT61 antisense transcripts in HeLa cells was confirmed by northern analysis of the mRNA fraction isolated from transiently transfected cells harvested 20–24 h post-transfection (data not shown). Differences in transfection efficiencies of the various antisense plasmids were

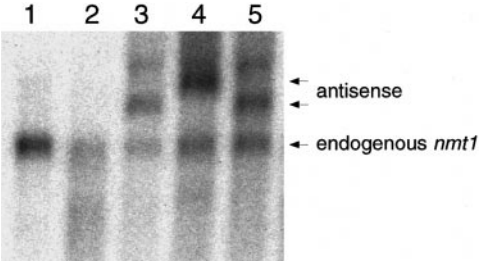

FIG. 3. Northern blot of total RNA isolated from *S. pombe* strain KC4-6 cells transformed with antisense RNA fragments expressed under the control of the *S. pombe nmt1* promoter from the pREP1 plasmid. The samples were hybridized with a 405-bp *BamH*I DNA fragment from the *nmt1* 3′ untranslated region. Lane 1, untransformed yeast; lane 2, pREP1 transformed yeast; lane 3, pGT-5′; lane 2, pGT-m; lane 5, pGT-5′.

TABLE 1

Relative Expression of mRNA Isolated from Transiently Transfected HeLa Cell

Plasmid	Size (bp)	Antisense/neomycin	Neomycin/GAPDH
pGT2	3475	0.17	0.11
pGT-5′	1211	1.04	0.08
pGT-m	1352	1.65	0.29
pGT-3′	1212	0.33	0.25
pGT61	379	0.26	0.14

Note. Northern data were quantified by phosphorimager and expressed as a ratio.

normalized using the relative expression levels of the neomycin mRNA from the pHOOK2 plasmids and the endogenous levels of GAPDH mRNA (Table 1).

In contrast to yeast, the levels of the different antisense mRNAs detectable after transient transfection of HeLa cells were variable when compared to the endogenous GAPDH mRNA (Table 1). This may reflect differences in stability of the various antisense RNAs between the two cell systems. The low levels of pGT2 detected in HeLa cells indicated there would be fewer transcripts available for suppression of the target gene and yet the highest reduction of β-galactosidase activity was observed for this construct (see below). This suggests that longer regions of complementarity may produce more stable duplexes as substrates for dsRNAse activities.

Antisense RNA suppression. In addition to the earlier studies in yeast, two new *lacZ* antisense RNA fragments (pGT-m, pGT-3′) were analysed and compared to the long 5′ (pGT-5′) previously used and designated pGT59 (5). Suppression of this gene was determined by a reduction in β-galactosidase activity as measured by a solution assay and comparison to the vector-transfected control. Analysis of the relative efficacies of these fragments (pGT-5′, pGT-m, pGT-3′) showed that suppression levels in fission yeast were approximately 20%, 24% and 23% respectively. If the 5′ antisense fragment (pGT-5′) was extended in a 3′ direction, to the point where it was complementary to the whole *lacZ* mRNA (pGT2), then suppression was approximately doubled to 45% (5).

To measure the efficacy of antisense RNA in HeLa cells, both the vector control or antisense plasmids and the target pSVβgfp plasmid were introduced by co-electroporation. The gfp fluorescence and β-galactosidase activity were measured and compared to the empty vector control. β-galactosidase activities were divided by gfp fluorescence to correct for the transfection efficiency of the target pSVβgfp plasmid. Values obtained for HeLa cells co-transfected with the antisense plasmids were expressed as a percentage of the values obtained for the cells co-transfected with the control plasmid (pHOOK2).

When the antisense plasmids were co-transfected with the target pSVβgfp plasmid into HeLa cells, all were able to suppress β-galactosidase activity when corrected for total fluorescence and compared to the empty vector control. When the suppression data was further corrected for the differences in transfection efficiencies of the antisense plasmids, all sequences were able to suppress β-galactosidase activity in a similar manner to that seen in *S. pombe* cells (Fig. 4).

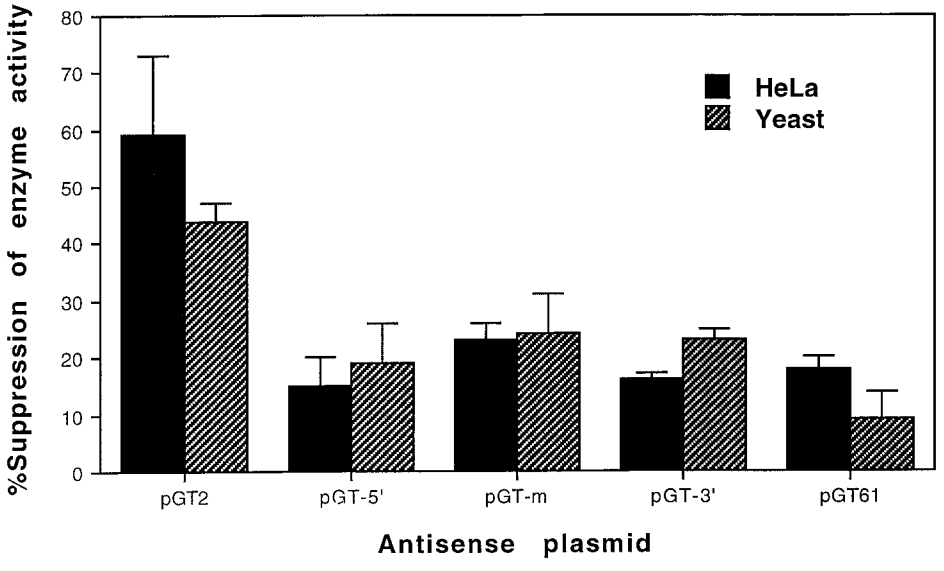

FIG. 4. Comparison of suppression levels of β-galactosidase activity in *S. pombe* and HeLa cells. Each bar of the histogram represents the average of three independent transformations or transfections. Each transformant/transfection was then assayed in triplicate for β-galactosidase activity. Standard deviations are indicated by the error bars.

DISCUSSION

This report describes experiments aiming to further the role of the yeast as model organisms for the study of artificial modes of gene regulation for application in human cells. There are many examples of the use of yeast to resolve biochemical, physiological and genetic pathways in mammalian cells and for this reason yeast have remained as important experimental microorganisms. Major reasons for this interest in yeast is the ease with which they can be handled, the ability to generate genetic mutants, the apparent conservation of many functions between yeast and mammalian cells, and the relative simplicity of the yeast genome. Not surprisingly, yeast have also become increasingly popular as tools for the development of several aspects of gene therapy. There are many examples in the literature describing the utility of yeasts to determine orphan gene function, however, the modalities of modifying gene function also have the potential to be studied and developed in yeast. In the present study, we address the question of whether fission yeast could be used as a rapid preliminary test system to identify antisense RNA sequences for eventual use in human cells. Fission yeast have been demonstrated to be good hosts for studying antisense function (3 and it was of interest to determine whether antisense sequences behaved in a similar manner in human cells as they did in yeast cells.

Rather than targeting endogenous genes, we established yeast and human cell protocols that involved expression of a near-identical gene target in each. The target *lacZ* gene fragment used was identical and transcription of this fragment was controlled by the SV40 early promoter in each case. This promoter has previously been shown to direct transcription of an mRNA molecule with an identical 5′ terminus in both fission yeast and human cells (11). Antisense RNA genes were introduced into both cell types on plasmids and expressed at levels in excess of the target mRNA levels. Yeast transformants harboured stable plasmids that were selected by complementation of prototrophic mutations. Plasmid introduction into HeLa cells was by transient transfection and steps were taken to ensure accurate measurement of key parameters. For example, target gene plasmid levels were normalized by comparison of green fluorescent protein levels produced by a *gfp* gene encoded on these expression vectors. Similarly, antisense plasmid levels were normalized by comparison of the theoretically invariant rates of transcription of the plasmid-borne neomycin phosphotransferase gene and the transcription of the chromosomally encoded GAPDH gene.

The results showed a remarkable correlation between all antisense fragments tested in both cell systems. For example, the most effective construct in HeLa and *S. pombe* cells was pGT2 with 59 and 44% suppression respectively of the target *lacZ* gene. This result could be interpreted in a number of ways. Firstly, the similarity of the efficacies could indicate a conservation of mechanisms of antisense function between the organisms. For example, if the mechanism is dependent upon an RNAse, then perhaps enzymes like the PacI gene product found in *S. pombe* play an analogous role to the RNAses found in mammalian cells (12). Alternatively, the mechanism could involve interference of nuclear export which is thought to have common features in the two cell types. Finally, the duplexes formed between the *lacZ* and antisense RNAs may have similar stabilities in each cell type and thus be equally effective in preventing translation.

Clearly fission yeast and mammalian cells are not identical and there are differences which may be expected to ensure that the observed results could not occur. For example, there are reports of fission yeast not having key proteins involved in RNA:RNA interaction such as an equivalent to the human hnRNPA1. However, it has been found that this protein did not alter antisense function when expressed in fission yeast, perhaps suggesting the existence of a compensating function if such proteins are essential for antisense function (D. Atkins, M. Patrikakis and B. Pontious, pers. commun.). In fact there are many aspects of fission yeast and mammalian cell RNA biology that argue in favour of a conservation of function. For example, there are similarities between fission yeast and human intron 5′ junctions (11). TATA contexts, snRNA sequences and structures (13) and promoter function (14).

This is the first report of the comparison of antisense function between two widely divergent eukaryotes and is a good indication that *S. pombe* can be used to predict the behaviour of antisense RNA in higher eukaryotes. It will be of interest to compare the precise mechanism and to determine whether similarities can be found in the biochemistry of antisense function in mammalian and yeast cells. Furthermore, a comparison of suppression of endogenous target genes, for example the highly conserved cell cycle genes, will shed light on how broad the similarities observed here will be. It is of interest to note that to date, no antisense function has been described in the baker's yeast *Saccharomyces cerevisiae*. This may reflect the degree of divergence between *S. pombe* and *S. cerevisiae* (15). Perhaps there is an enzyme activity conserved between fission yeast and human cells that is essential for antisense activity but is absent in the baker's yeast?

This result now provides encouragement for the further development of fission yeast as a host in which to develop gene therapy agents such as antisense and ribozyme RNAs. The potential of using yeast for screening experiments is well established and the increasing number of human gene homologues emerging

in yeast sets the scene for the development of genetic methods as tools for use in the field of gene therapy.

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