Short Technical Reports

system or even by eye. As compared with conventional cDNA RDA, which is highly specific to isolate a small number of differentially expressed genes, our technique facilitates the study of the expression patterns of arrayed cDNA clones or whole libraries. We are convinced that in the future, hybridizations with cDNA-RDA probes and gridded library arrays will represent a valuable technique to study differential gene expression in a variety of biological systems without needing to purchase expensive equipment.

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M. Geng, C. Wallrapp, F. Müller-Pillasch, M. Frohme¹, J.D. Hoheisel¹ and T.M. Gress
*University of Ulm
Ulm, and
¹Deutsches Krebsforschungszentrum
Heidelberg, Germany*

Fission Yeast Expression Vectors Adapted for Positive Identification of Gene Insertion and Green Fluorescent Protein Fusion

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ABSTRACT

A pYZ series of fission yeast expression vectors, derivatives of the pREP series, was designed to allow positive identification of cloned gene insertion and fusion to the green fluorescent protein (GFP) gene for *in vivo* analysis of gene expression. To validate this new vector system, the human immunodeficiency virus type 1 (HIV-1) vpr gene of viral isolate pNL4-3 was expressed in the pYZIN vector. Vpr-induced phenotypic changes were the same as those observed with vpr expressed from pREP1N. Consistent with observations in mammalian cells, a Vpr-GFP fusion protein localizes on the nuclear membrane of fission yeast cells. Additionally, we were able to detect a naturally occurring mixture of vpr genes from a plasma sample of an HIV-infected pediatric long-term surviving patient. These pYZ vectors expedite gene cloning for general purposes and are particularly suited for large-scale random gene screening.

INTRODUCTION

The pREP series of expression vectors (11,12) is widely used in fission yeast research. The advantageous feature of these vectors is the thiamine repressible *no message in thiamine* (*nmt1*) promoter, which is useful for demonstrating gene-specific effects in media either containing or lacking thiamine. Furthermore, the effect of gene dosage can also be tested, since the set of three expression vectors, pREP1, pREP41 and pREP81, have different sequences in the TATA box and consequently express the same mRNA at different levels (1,6). The pREP vectors have been modified for various purposes. The pRIP series were derived from these vectors by deleting the *arsI* origin of replication sequence and are

Short Technical Reports

used for the creation and expression of a single-copy gene integrated into the chromosome (12). Other modifications of these vectors include pREP-X vectors that lack an ATG start codon (5), pSLF/pDS vectors that allow hemagglutinin (HA) or glutathione S-transferase (GST) taggings (7) and the pSGA vector for green fluorescent protein (GFP) fusions (16). The pYZ series described here is adapted for positive identification of cloned gene insertion, fusion to the GFP and large-scale random gene screening.

MATERIALS AND METHODS

Construction of pYZ vectors is described in the Results and Discussion section. The same strategy was used to clone and express the *vpr* gene of viral

isolate pNL4-3 in the pYZ vectors as was used previously for the pREP vectors (18). Human immunodeficiency virus type 1 (HIV-1) *vpr* genes isolated from an infected long-term survivor were also cloned and expressed in pYZ1N in this study. The long-term survivor was defined as more than eight years of age, perinatally infected, relatively high and stable CD4 counts, no opportunistic infections, asymptomatic and non-AIDS status. To clone the *vpr* genes from the patient's specimen, the viral RNA was first extracted from plasma using a QIAmp® Viral RNA Kit (Qiagen, Chatsworth, CA, USA), reverse-transcribed to cDNA and then polymerase chain reaction (PCR)-amplified using the *rTth* RT PCR Kit fol-

lowing manufacturer's instructions (Perkin-Elmer, Norwalk, CT, USA). The 340-bp *vpr* gene fragment was amplified by nested PCR using VPR-ext1 (5'-GCAGGACATAACAAGGTAGG-ATCTCTAC-3') and VPR-ext2 (5'-GGTACAAGCAGTTTTAGGCTGAC-TTC-3') primers for first-round PCR and VPR-Nde (5'-GAGGACATATGG-AACAAGCCCCAGAAGACC-3') and VPR-Bam (5'-CAGTTGGATCCTGAC-CTTCCTGGATGCTTCCAG-3') primers for second-round PCR. The amplified DNA fragment was then digested with *Bam*HI and *Nde*I enzymes and ligated to the new fission yeast expression vectors digested with the same enzymes. The ligation product was transformed into *Escherichia coli* DH5α™ for identification of inserted *vpr* genes by formation of white colonies on 5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside (X-gal; Life Technologies, Gaithersburg, MD, USA)-containing agar plates. The complete nucleotide sequence of the *vpr* inserts in pYZ1N was determined with the ABI Model 377 Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA). For Vpr nuclear localization, GFP was fused in-frame at 5' end of the *vpr* gene by the introduction of a *Sac*II site during PCR and cloning at the *Sac*II and *Bam*HI sites of the pYZ3N-GFP vector. Nuclear localization of Vpr in fission yeast cells was examined using fluorescent microscopy with propidium iodide staining of nuclei (16). The plasmid DNA was transformed into the wild-type SP223 *Schizosaccharomyces pombe* cells (h-*ade*6-216 *leu*1-32 *ura*4-294) by the lithium acetate procedure (13,19).

Figure 1. Maps of the pYZ vectors. (A) pYZ1N contains the wild-type *nmt1* promoter, whereas pYZ41N and pYZ81N contain attenuated *nmt1* promoters with mutations in the TATA box. (B) pYZ3N-GFP contains the wild-type *nmt1* promoter and is a derivative of pKS406, which is the same as pSGA (16) except that it has GFPmut2, a brighter version of GFP (2). The α -peptide of β -galactosidase was inserted as a *Not*I-*Bgl*III fragment into the *Not*I and *Bam*HI sites of pKS406 to delete most of the original linker sequence of pKS406. Unique cloning sites in these vectors are indicated. In-frame fusions to GFP can be made at the *Sac*II or *Not*I sites of pYZ3N-GFP. *ars*1, origin of replication from *S. pombe*; *Leu*2, *Saccharomyces cerevisiae* leucine biosynthesis gene; *Amp*^R, bacterial ampicillin-resistance gene.

RESULTS AND DISCUSSION

We have modified the pREP vectors by inserting an *E. coli* α -peptide (position 239–684 on the pUC19 plasmid) of the *lacZ* (β -galactosidase) in the opposite orientation to the *nmt1* promoter (Figure 1). Expression of the α -peptide complements the *lacZ*ΔM15 deletion in *E. coli* strains such as DH5α or JM105 (3). Unidirectional insertion of the gene of interest at the *Nde*I site (containing the ATG start codon), and one of the unique cloning sites results in removal

Short Technical Reports

of most of the α -peptide coding region of the vector, producing colorless, rather than blue, colonies on medium containing X-gal. Most importantly, since the distance between the *nmtI* promoter and the ATG start codon remains the same as in the pREP vectors, the promoter strength is unchanged. In this arrangement, we are able to select for a gene insert in *E. coli* and express the insert at the same strength as the original pREP *nmtI* expression system in *S. pombe*. The new vectors corresponding to pREP1, pREP41, pREP81 and pSGA are designated as pYZ1N, pYZ41N, pYZ81N (in which N represents an additional *NotI* site) and pYZ3N-GFP, respectively (Figure 1). Complete nucleotide sequences of these vectors have been submitted to GenBank® (Accession Nos. AF049063, AF049064, AF064066 and AF064067).

To validate this new vector system, we expressed the *vpr* gene of viral isolate pNL4-3 from these vectors in fission yeast cells. Our early studies showed that expression of this *vpr* gene in pREP1N induces cell morphological changes, cell-cycle G2 arrest and cell death (18,20). Expression of the *vpr* gene from the pYZ1N vector had effects on cell morphology, cell-cycle G2 arrest and cell killing, which were indistinguishable from expression of *vpr* from the pREP1N vector (18,20). Furthermore, the extent of these Vpr-induced cellular effects in pYZ vectors correlates with the levels of expression when *vpr* is expressed in pREP1N, pREP41 and pREP81 (20). Twenty-four hours after *vpr* gene induction, the relative *vpr* mRNA levels expressed from these vectors and measured by a real-time PCR method (8) are 1, 0.3 and 0.04, respectively.

In mammalian cells, Vpr localizes to the nucleus (4,10). To test whether Vpr localizes to the nucleus of fission yeast cells, the *vpr* gene was fused to GFP in the pYZ3N-GFP vector and expressed in the fission yeast cells. The GFP-Vpr fusion protein has effects similar to those of the Vpr protein alone, with the fusion protein inducing morphological changes, G2 arrest and cell death in fission yeast (data not shown). For the nuclear localization studies, we also expressed GFP alone as a control. No fluorescent signals were detected with

either construct in gene-repressing, thiamine-containing media (data not shown). GFP protein produced from the control plasmid in thiamine-free medium was dispersed throughout the cells and observed in both the cytoplasmic and nuclear compartments (Figure 2, a and b). It is noticeable that some cells contain strong GFP spots that are not associated with the nuclei. In contrast, the GFP-Vpr fusion protein was localized specifically on the nuclear envelopes (Figure 2, c–e), which is identical to the localization of Vpr in mammalian cells (4,10).

Results of our studies and others suggest that the effects of Vpr on cellular morphology, G2 arrest, cell killing and nuclear localization are highly conserved between fission yeast and human cells (9,14,15,18,20). As Vpr plays an important role in HIV-1 pathogenesis, we are interested in using fission yeast as a model system to study the relevance of particular Vpr functions to HIV pathogenesis. However, since HIV normally exist as a mixture (quasi-species) in a given patient specimen, *vpr* genes representing different viral variants of a population need to be cloned and tested for their functions. One obstacle to using pREP vectors for such a large-scale molecular cloning task is that they lack a positive identification marker for recombinant inserts. To test whether we could detect *vpr* variants in a naturally occurring viral population, *vpr* genes were amplified by PCR and cloned in pYZ1N from a blood sample of an infected pediatric long-term survivor, as described in Materials and Methods. Twenty-one independent white colonies were randomly chosen, from which *vpr* genes were sequenced. This sequencing confirmed that all 21 plasmids contained *vpr* inserts and identified three *vpr* sequence variants. One *vpr* variant appeared to be the predominant variant (48%, 10/21) in

Figure 2. Expression of GFP alone (a and b) and GFP-Vpr (c–e) in fission yeast cells. (a and c) GFP fluorescence; (b and d) corresponding images of nuclei staining by propidium iodide. (e) Image was photographed under combined fluorescent excitation and transmitted white light to show cell shape and the relative location of the nucleus to cell membrane.

Short Technical Reports

this population, which was confirmed by a direct sequencing of the PCR product (data not shown). The other two *vpr* variants represented 38% (8/21) and 14% (3/21) of the plasmid population. Interestingly, the mutations differing among these 3 variants were clustered in the region of amino acids 83–85. These carboxyl-terminal mutations suggest potential defects in the ability of Vpr to induce cell-cycle G2 arrest as being linked to the slow disease progression of infected patients (17).

In summary, we describe a pYZ vector series that can be used to expedite molecular cloning for general purposes or large-scale random gene cloning. To validate this system, we demonstrated that Vpr induces the same phenotypic changes when expressed from pREP and from the new pYZ vectors. In com-

bination with the power of automated DNA sequencing and the assays for Vpr function in fission yeast developed in our laboratory (18,20), this rapid cloning system offers us an unique opportunity to screen a large number of *vpr* genes from patients and assess the relevance of particular Vpr functions to HIV pathogenesis.

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Yuqi Zhao^{1,2}, Robert T. Elder¹, Mingzhong Chen¹ and Jian Cao¹

¹Children's Memorial Institute of Education and Research,

²Northwestern University Medical School
Chicago, IL, USA