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A directed DNA sequencing strategy based upon Tn3 transposon mutagenesis: application to the *ADE1* locus on *Saccharomyces cerevisiae* chromosome I

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ABSTRACT

We have developed a directed DNA sequencing strategy based upon the *Escherichia coli* transposon Tn3. This transposon displays little sequence specificity for transposition and is thus well suited to this task. Both mini-Tn3 transposons and sequencing vectors bearing the phage f1 single stranded origin of replication have been constructed. Upon mutagenesis of a target sequence, a population is produced in which each clone has two f1 origins of replication, one of which is at a variable position depending upon the tranposon insertion site. When helper phage is added to the mutagenised population, the two f1 origins present on each clone are nicked, dividing the packaged strand into two segments, each of which is packaged into a separate phage particle. One of these segments contains no resistance markers and is lost, whilst the other is recovered as a deleted clone with a single chimeric f1 origin. A unidirectionally, variably-deleted set of sequencing clones is produced, and appropriately sized clones are sequenced using a primer complimentary to the transposon end. In addition to being inexpensive, the method does not require the same degree of technical expertise needed for many *in vitro*, enzymatically based methods. The strategy has been used to determine 2.6 kilobases of nucleotide sequence in the *Saccharomyces cerevisiae ADE 1* locus.

INTRODUCTION

Directed DNA sequencing strategies often involve the generation of overlapping deletions, made in a progressive, enzymatically-based manner from one end of a population of linear DNA fragments, such that after recircularisation a different sequence in each clone lies next to an invariant priming site (1, 2, 3). The position in the fragment which will be sequenced is related to the size of the deletion, and hence to the overall size of the clone. Thus, one can sequence a given region by selecting an appropriately sized clone. Other directed strategies exploit DNAse 1 nicking activity (4) or Tn9 transposon insertion (5), but are relatively sequence specific and are not in common use. These directed strategies are designed to minimise the amount of redundant sequence data that is generated, but are usually technically complex and labour intensive. Also, the underlying enzymologies have inherent sequence specific biases that can result in the underrepresentation of certain size classes of clones.

The Tn3 transposon family, which includes Tn3 and gamma delta, displays relatively little insertion site specificity compared to other transposons. This property can be exploited in the development of DNA sequencing strategies. Directed strategies based upon the *in vivo* transposition of the transposon gamma delta have recently been reported. These strategies use restriction digestion and Southern hybridisation (6), and the polymerase chain reaction (7) to map insertion events. We present here a low cost and simple directed strategy that is based on the transposon Tn3. The strategy is based upon transposon insertion followed by an *in vivo* site-specific deletion that generates packaged, single-stranded DNA clones of different sizes. Briefly, transposon mutagenesis is first carried out in *Escherichia coli* by a previously described, simple and effective procedure (8). A deletion event is then catalysed *in vivo* between two phage f1 origins of replication, one on the transposon and one based on the target plasmid, by taking advantage of the nicking activity at those origins during replication and packaging of single-stranded DNA (9). The size of the deletion is dependent upon the transposon insertion site. Deleted clones are then directly assayed for size by agarose gel electrophoresis of undigested, packaged, single-stranded DNA, and appropriately sized clones are sequenced from a priming site at the transposon terminus. The advantages gained by this system over the mapping strategies employed in the gamma delta systems (6, 7) are that it does not necessitate either DNA purification, restriction digestion and Southern hybridisation or the use of a polymerase chain reaction (which can be unreliable for larger fragments) to map insertion sites in identically sized molecules. Instead, the generation of a deletion *in vivo* allows direct size assay by agarose gel electrophoresis to map transposon insertion sites. This method, as employed in other single-stranded strategies, is inexpensive, quick and reliable.

We have applied this 'Tn3 deletion' sequencing strategy to a DNA fragment encoding the *Saccharomyces* genes *ADE1* and part of *FUN2* (10).

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MATERIALS AND METHODS

Construction of vectors and transposons

Construction of pHSS6/f1: The f1 origin from pUC f1SH-K (a gift from H. Fried) was subcloned as a Sma I/Hpa I fragment into the pHSS6 (8) Sma I site. Transformants were picked and tested for packaging function, and then the orientation of the f1 origin was determined by restriction analysis. Construction of pUNC9(+) and pUNC9(−): The Not I polylinker fragment in pHSS6/f1 was replaced with the Not I α lacZ and pUC9 polylinker fragment from pHSS8 (11). The transformants were screened for α complementation, restriction digested and sequenced using universal primer (12) to determine the two orientations for pUNC9(+) and 9(−). Construction of pUNC19(+) and pUNC19(−): The Eco RI/Hind III polylinker fragments from pUNC9(+) and 9(−) were replaced with the corresponding fragment from pUC19. Construction of mini-Tn3 UFO: The f1 origin fragment (above) was cloned into the end-repaired Eco RI site of m-Tn3 URA3 (8) and tested as above. Construction of mini-Tn3 FOα: The f1 origin fragment (above) was cloned into the end-repaired Hind III site of m-Tn3. The end-repaired Not I fragment from pHSS8 (the α lacZ/polylinker fragment) was then cloned into the end-repaired Eco RI site of this construct and tested as above. The Klenow fragment of *E. coli* polymerase I was used for all end repairs.

Construction of the helper phage M13CO7

The helper phage M13CO7 is a derivative of the kanamycin resistance-conferring helper phage M13KO7. A mini-Tn10 transposon (13), conferring chloramphenicol resistance to the host strain, was transposed into M13KO7 and the packaged supernatant was selected for the ability to transduce this marker into a host strain. Individual transductants were then screened for kanamycin resistance. Sensitive transductants were assumed to harbour a Tn10d insertion into the kanamycin resistance gene, inactivating it. Insertions of this type were recovered with a high frequency, since this is one of the few non-essential regions in M13KO7 (when not under selection for kanamycin resistance).

Source of the test sequence

A 3.0 kb Not I fragment encoding the *S. cerevisiae* gene *ADE1*, part of *FUN2* and some pBR322 sequence (10) was subcloned from pHSS4/*ADE1* (8) in both orientations into pHSS6/f1 to create the plasmids pHSS6/f1/*ADE1*.13 and pHSS6/f1/*ADE1*.15.

Transposon mutagenesis

Transposon mutagenesis was carried out as previously described, using the *E. coli* RecA⁻ strains described therein (8,11), with some modifications (Figure 1, A−D). The procedure is described here for a single transformant: The plasmid pHSS6/f1/*ADE1* was transformed into an F− strain containing pLB101, the source of the Tn3 transposase. A 2ml overnight culture of a transformant was grown in 2YT (chloramphenicol 12.5μg/ml, kanamycin 25μg/ml). Similarly, a 2ml overnight culture of an F+ strain harbouring the transposon based on a conjugative plasmid was grown (in 2YT, ampicillin 100μg/ml). The next day, the two strains were mated to transfer the transposon to the cell harbouring the target DNA: A 100μl aliquot of each culture was diluted 1:20 into fresh media without antibiotics in a 15ml tube and grown for 2 hours at 37°C with shaking. The cells were allowed to mate by combining the contents of the two tubes and incubating at 37°C for 30 minutes without shaking. A 1μl aliquot was then diluted to 100 μl in fresh medium and immediately plated out (chloramphenicol 12.5μg/ml, kanamycin 25μg/ml, ampicillin 100μg/ml) and incubated at 30°C overnight to allow transposition and cointegrate formation to occur. At the same time, a 2ml overnight culture of the *E. coli* strain NS2114Sm (the source of *cre* recombinase) was prepared (in 2YT, streptomycin 100μg/ml). The next day, the approximately 1000 to 2000 cointegrate-containing colonies were resuspended in 2ml of medium. A small aliquot of the suspension was added to 2ml of liquid medium such that the turbidity appeared to be approximately the same as that of a 1:20 dilution of the NS2114Sm culture. These dilutions were then grown and mated as above, and an aliquot plated out (kanamycin 25μg/ml, ampicillin 100μg/ml, streptomycin 100μg/ml) and incubated overnight at 37°C. The colonies seen the next day were assumed to each represent an insertion of the transposon into the target sequence. In practice, this mutagenesis was carried out separately and in parallel for at least two individual transformants from each orientation of the *ADE1* insert in the target plasmid. At the very least then, each plate carried a different profile of insertion events, and at best each colony represented a unique insertion event.

Generation of deletions

Colonies from the final step of the transposon mutagenesis were pooled in liquid media and vortexed thoroughly (Figure 1, D−F). A 1ml aliquot was frozen at −70°C as a 20% glycerol stock, and another aliquot was grown in liquid media (kanamycin 25μg/ml, ampicillin 100μg/ml, streptomycin 100μg/ml) at 37°C until mid log phase. At this point the helper phage M13CO7 (and chloramphenicol, 12.5μg/ml, 20 minutes later) were added, and the culture was sampled at 2, 4 and 12 hours (or overnight) post infection. Samples were immediately microfuged and the supernatant incubated at 65°C for 15 minutes to kill live cells.

Reinfection, assay of clones and template preparation

Supernatants containing deleted clones were reinfected into the *E. coli* strain DH5 α F′ at low titer to give isolated colonies. Colonies were picked, and single-stranded packaged phagemid was prepared as previously described (12) and directly assayed for size by agarose gel electrophoresis. Single stranded template DNA was prepared either from 1.5ml cultures (14) or from 240μl cultures grown in 96-well microtitre plates (15,16).

DNA sequencing

Suitably-sized templates were sequenced with an oligonucleotide complementary to the 3′ end of the β-lactamase gene on m-Tn3 (5′ AATCTCATGACCAAAATCCC 3′), using the dideoxy chain-termination method (17) and buffer gradient acrylamide gels, following the protocols of Bankier et al. (14).

Sequence analysis computer programs

DNA sequence was compiled and analysed using the Staden DBSYSTEM programmes (18), and further analysed using the GCG program package version 6.2 (19). Database searches were performed using the FastA algorithm of Pearson and Lipman (20) on the most recent version of Genbank available by electronic mail (November, 1990).

RESULTS

Rationale of the 'Tn3 deletion' sequencing strategy

The transposon *E. coli* Tn3 typically encodes both the sequences necessary for transposition and an accessory gene such as β-lactamase, encoding ampicillin resistance. Tn3 displays no strong

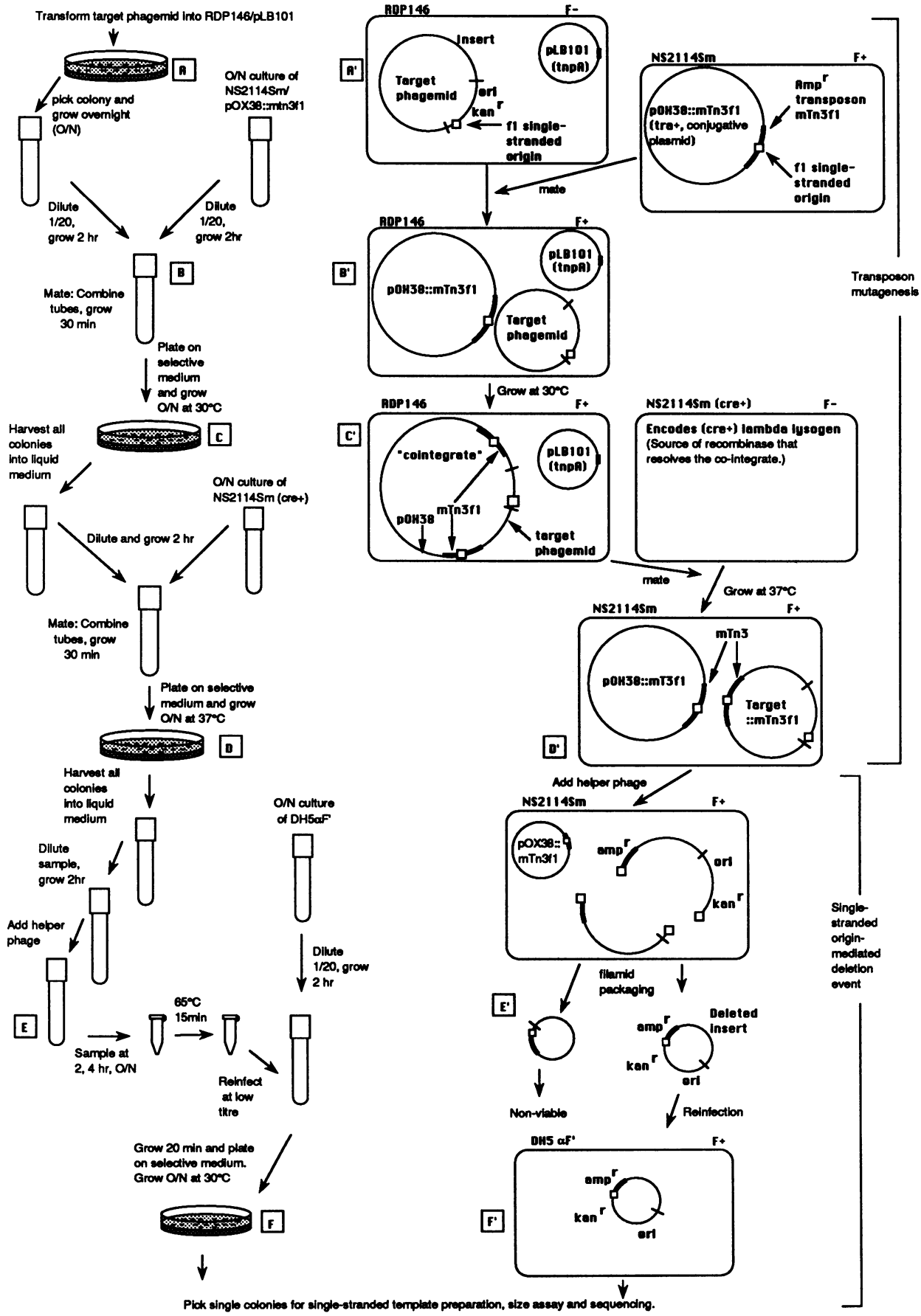

Figure 1. Overview of Tn3-deletion sequencing. Methodology is shown to the left, corresponding molecular events to the right.

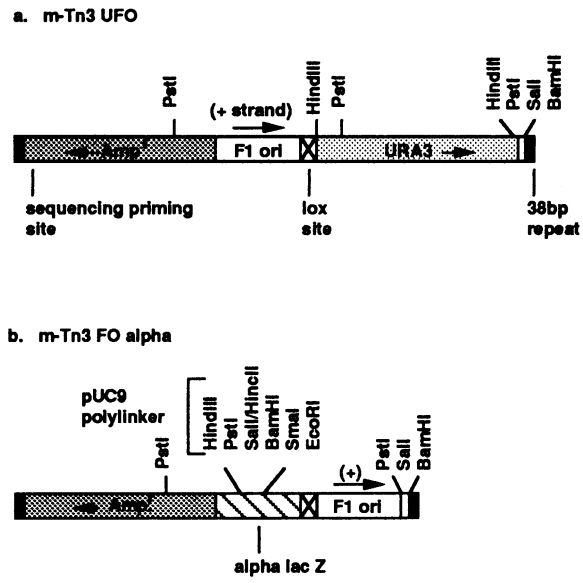

Figure 2. Mini transposons used in Tn3-deletion sequencing.

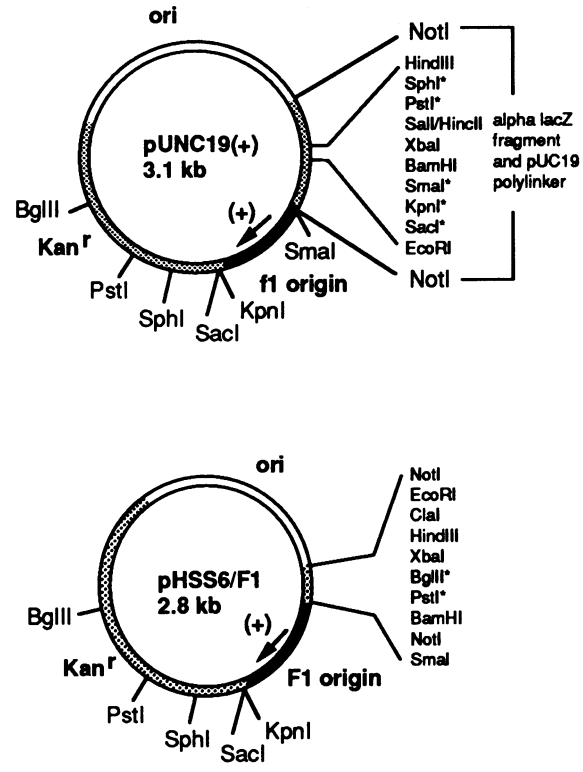

Figure 3. Target vectors used in Tn3-deletion sequencing. * denotes sites in polylinker present more than once in vector.

insertion site specificity, although it displays a mild regional specificity for adenosine and thymidine rich sequences. Other transposons, such as Tn5, 9 and 10, are more prone to insertion site specificity (21).

The sequencing strategy presented here is a development of the Tn3-based transposon mutagenesis method described by Seifert et al. (8), which efficiently produces a large number of mutagenised clones, each with an effectively randomly placed transposon insertion in the target sequence, and with very little background of non-useful insertion events such as multiple insertions or insertions into the *E. coli* genome. All mutated clones carry a complete copy of the target sequence disrupted by a single, randomly-placed insertion of mini-Tn3, and are thus identical in size. While quite complex in theory, the method is in fact simple to execute. (See Figure 1, A–D and method section.)

We have constructed both mini-Tn3 transposons and target vectors that carry an f1 phage single-stranded origin of replication (figures 2 and 3). The target vectors are thus in effect phagemids. After transposon mutagenesis using these constructs a molecule with two f1 origins is created, which is exploited as follows to create a site-specific deletion: When the single-stranded phages M13 and f1 replicate, the packaged strand is nicked at the origin of replication. A molecule carrying two of these single-stranded origins is nicked at *both* of these sites during replication, and is cleaved into two pieces which recircularise and are packaged independently (9). Presumably both pieces of the parent give rise to packaged material, but only that piece which encodes the ampicillin and kanamycin resistance determinants and the bacterial replication origin is recovered. Thus, a deletion is made in each mutagenised phagemid, the size of which is determined by the transposon insertion site. The deleted DNA contains non-essential vector and transposon sequences, and a piece of target DNA located in between. Insertions into vector DNA inactivate or result in the deletion of essential sequences and are not recovered. A randomly mutagenised population, therefore, gives rise to a randomly-deleted population can be used to sequence a fragment of DNA in a directed manner.

It should be noted that the mini-Tn3 can insert in either orientation, but only transposition events which place both f1 origins in the same orientation on a phagemid give rise to a successful deletion and packaging event. Insertions in the other orientation package very inefficiently or not at all, and also would not carry all of the determinants necessary for survival. Thus, deletions are made from one end of the target DNA for a given orientation in the vector, allowing each strand to be deleted and sequenced independently.

The deletion event is mediated by simply adding helper phage to a liquid culture of the mutant bank, and the packaged, deleted target molecules are harvested from the supernatant. These are then reinfected at low titer into a host suitable for single-stranded sequencing template production, and are plated out onto appropriate selective media. Individual clones are picked, helper phage added, and packaged phagemid from the supernatants assayed for size by agarose gel electrophoresis. Suitably sized clones over the extent of the observed size range can be sequenced using an oligonucleotide primer complementary to the end of the mini-Tn3, allowing sequence in the target DNA to be read.

Vector and transposon design

Several vectors and mini-Tn3 transposons were constructed during the development of the method (figures 2 and 3). The vectors are all kanamycin resistant, carry an f1 origin of replication in the necessary orientation, and lack a copy of the Tn3 38 base pair (bp) terminal repeat that is closely linked to β-lactamase in most vectors and which confers immunity to Tn3 transposition. The vectors' small size (approx. 3 kb) allows them to carry large inserts. pUNC9(+), 9(−), 19(+) and 19(−) carry the α lacZ fragment and thus allow easy cloning by screening

for white colonies on X-gal medium, at the expense of providing a little more target DNA that does not yield useful sequence.

Mini-Tn3 transposons carrying f1 origins in the required orientation were constructed. m-Tn3 UFO is of use when one wishes to reintroduce a disrupted clone into *S. cerevisiae*. m-Tn3 FOa carries the a lacZ fragment from pUC9 which is retained after the deletion event, and allows subsequent manipulation of the remaining target sequence (see discussion).

Construction of the helper phage M13CO7

The kanamycin resistance-conferring helper phage M13KO7 packages phagemid with high efficiency, but is not the helper phage of choice for phagemids that also encode kanamycin resistance. The interference-resistant phage R408 was also found to be unsuitable. We have disrupted the kanamycin resistance gene of M13KO7 with a defective Tn10 transposon (13) that carries the chloramphenicol resistance gene, to create a chloramphenicol resistant, kanamycin sensitive helper phage designated M13CO7. It is this phage that we have used to produce the deletions in the mutated target vectors, and also to package the deleted clones for template preparation. We have found this helper phage to have the same beneficial packaging characteristics as M13KO7.

Test sequencing project

The Tn3 deletion sequencing strategy was applied to a 3.0 kb fragment encoding the *S. cerevisiae* gene *ADE1* (phosphoribosyl-amino-imidazolesuccinocarboxamide synthetase [EC 6.3.2.6]), which functions in the adenine biosynthesis pathway, and part of *FUN2*, a non-essential gene of unknown function (10). The fragment was subjected to transposon mutagenesis, M13CO7-mediated deletion, reinfection, assay for size, and sequencing in both orientations as described in 'materials and methods' and outlined in figure 1.

Size distribution of clones produced during the sequencing of the *ADE1* locus

A total of 102 clones were sequenced to determine the complete nucleotide sequence of this fragment on both strands, with an average of 5.6-fold redundancy per base. 78 of these were on the [+] strand (i.e. same sense as *ADE1* mRNA), and 24 were on the [−] strand. All clones chosen for sequencing were sequenceable with the β-lactamase primer. Additionally, all of the clones sequenced were of the correct size on the agarose gel when compared to their predicted size from the sequence data, demonstrating the efficiency and faithfulness of the deletion method. To show the random clone-size distribution that can be obtained, 40 clones from the [+] strand were randomly picked and sized by agarose gel electrophoresis (figure 4). Calculation of the sizes of these clones (based on migration distance and sequence data) shows that in all probability the complete sequence could be determined from this population, requiring gel readings of approximately 250 bp and one long reading of 400 bp.

On the [+] strand, many clones of the same apparent size were intentionally sequenced, so that possible biases in the distribution of clones could be analysed. It was found that clones of the same apparent size on an agarose gel were sometimes a few base pairs different in size, and were sometimes identical. Identical clones could arise from two separate insertions into an identical position, or could be sibling clones from the same insertion event.

The [−] strand of this clone suffered from a sequence-related artefact that resulted in reduced yields of packaged phagemid.

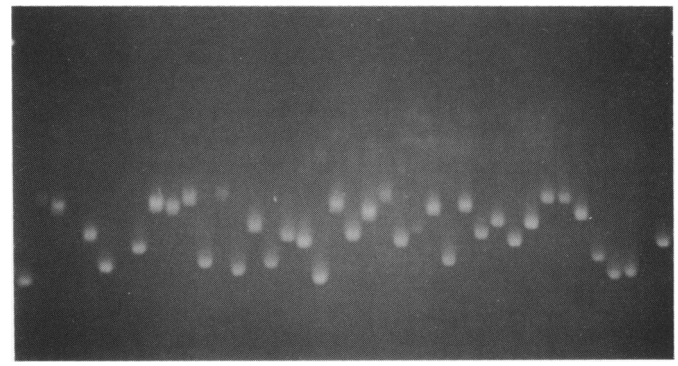

Figure 4. Agarose gel fractionation of single-stranded clones bearing transposon/f1 origin-mediated deletions in a 3.1 kilobase *ADE1*-encoding fragment. Selected clones were sequenced to confirm that deletions were over the full range of the fragment. Vector sequences remaining are 3.7 kb in size, hence the largest (upper) clones are approximately 6.8 kb, and the smallest 3.8kb.

Artefacts of this type are encountered occasionally with all single-stranded vectors, and may be solved (or not!) by changing the vector, insert orientation, helper phage or host strain. In the case of the sequence reported here, the artefact was manifested in the following manner: 75% of the deleted clones picked and sized by agarose gel electrophoresis were small, with approximately 80% or more of the sequence deleted, and gave a normal DNA yield (as determined by ethidium bromide staining). The remaining clones were evenly distributed in size, with between 0 and 80% of the clone deleted, but were of relatively poor DNA yield (data not shown). We surmise that a sequence in the target DNA, located at a position approximately 80% along the fragment, was inhibitory to the packaging of single stranded phagemid in this orientation, and that only clones in which this sequence was deleted produced normal titers of phagemid. During the en masse generation of deletions, therefore, clones which deleted 80% or more of the target sequence would also delete this inhibitory sequence, would be packaged in higher titers than clones giving rise to smaller deletions, and consequently were picked more frequently when choosing clones for size assay. This bias, then, can be attributed not to transposon specificity but to a packaging artefact caused by the sequence of the ADE1 insert. We have not encountered a similar artefact in clones sequenced subsequently by this method. Despite this problem, size assay of 192 colonies picked and grown in 96-well microtiter plates yielded enough suitably-sized clones to determine the complete the sequence of the [−] strand. Contiguous sequence in this orientation was obtained from 24 clones, emphasising the advantage of a directed approach.

Sequence analysis of the *ADE1* locus

The nucleotide sequence of the single copy *ADE1* locus and the predicted protein sequences of the long open reading frames (ORFs) corresponding to *ADE1* and *FUN2* are shown in figure 5. This sequence is available in Genbank under the accession number M67445.

The only complete, long ORF in the fragment exists at the position in the locus shown previously to encode *ADE1* (8). The predicted protein sequence is 306 amino acids (a.a.) in length. This sequence was compared to the NBRF protein and the translated Genbank databases and was found to bear most homology to the *Bacillus subtilis* and *E. coli* enzymes of the same

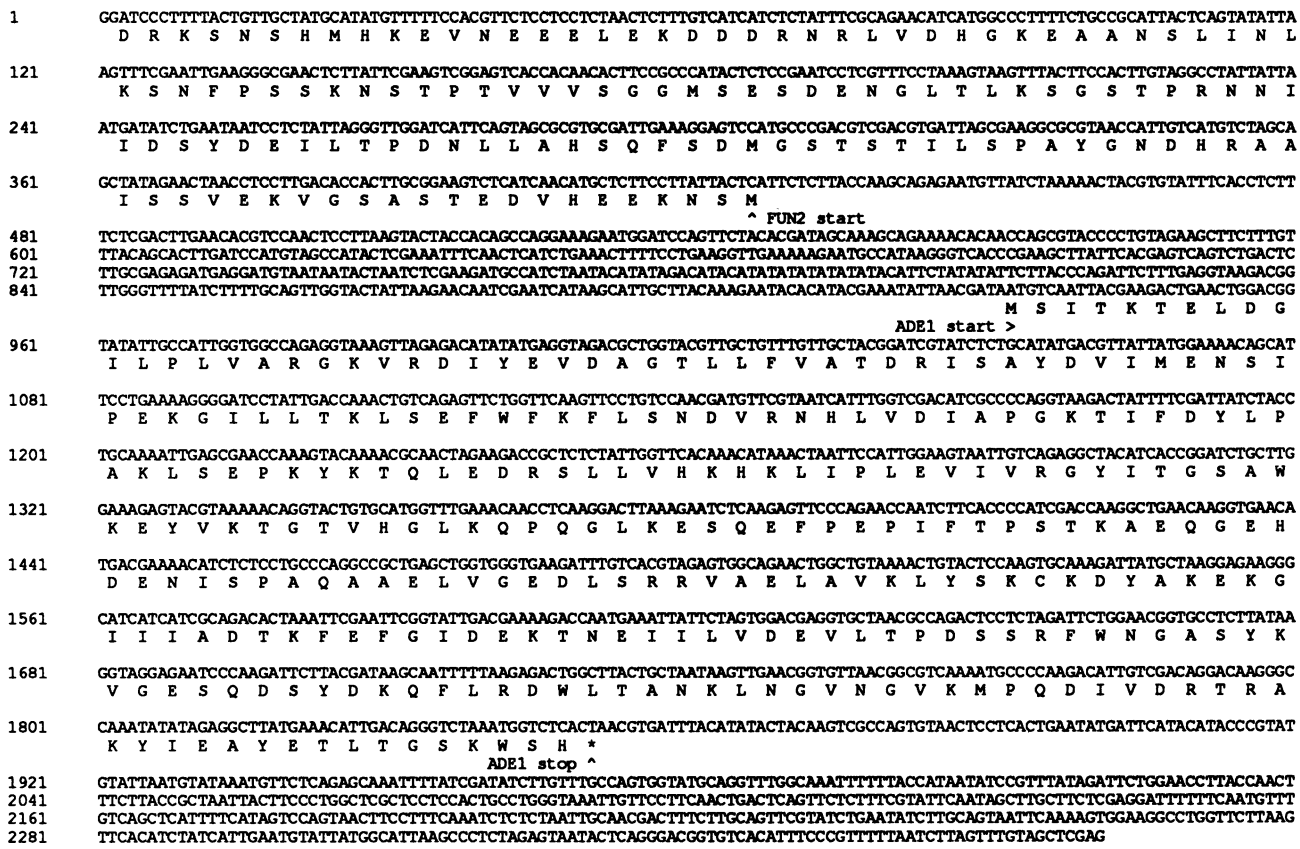

Figure 5. Nucleotide sequence of the *S. cerevisiae ADE1* locus on chromosome I, including partial sequence of the *FUN2* gene.

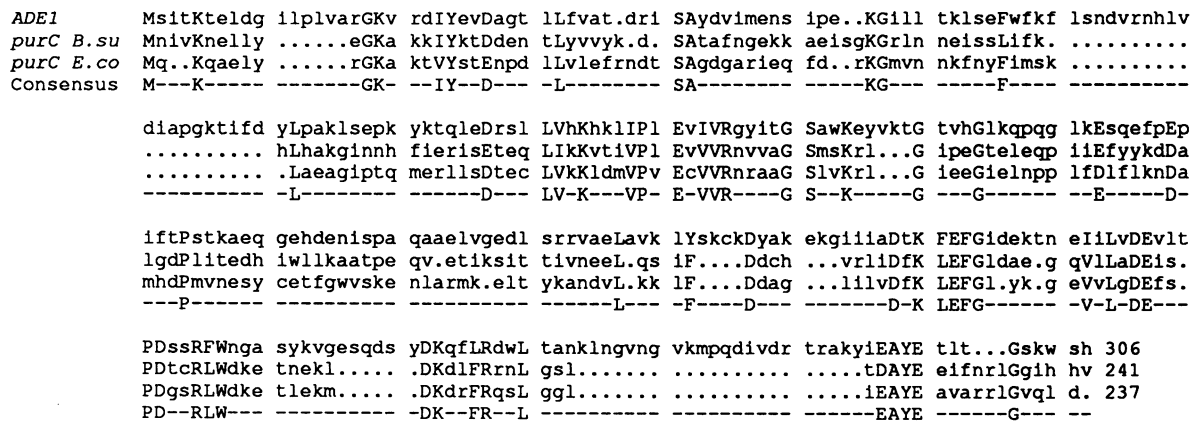

Figure 6. Alignment of the phosphoribosyl-amino imidazole succinocarboxamide synthetase [EC 6.3.2.6] homologues *ADE1* (*S. cerevisiae*), *purC* (*B. subtilis* [*B.su*]), and *purC* (*E. coli* [*E.co*]).

name and function, encoded by the *purC* genes (22, 23). A composite alignment, optimised manually, is shown in figure 6. Several motifs appear to be conserved, presumably due to their recognition of common substrates. We assume, based upon this alignment and primer extension extension studies on *ADE1* mRNA (data not shown), that the ATG codon at nucleotide position 932 (figure 5) is the start codon used. Nucleotides 701 to 2147 (encompassing the *ADE1* coding sequence) correspond to previously sequenced *S. cerevisiae ADE1* (24) which recently became available in the Genbank database (13 September 1990, accession number M38331). The two sequences differ at seven nucleotide positions, possibly due to polymorphic differences between the two genes. Four of these lie within the ADE1 coding region, of which three are silent. The fourth gives rise to a conservative amino acid change in a region that is not conserved among *S. cerevisiae*, *B. subtilis* and *E. coli*.

The incomplete ORF corresponding to *FUN2* (starting at nucleotide position 428, 5′ to *ADE1*, and on the [−] strand) was

also translated and used to search the NBRF protein database. The best match found was to folate hydrolase (carboxypeptidase G2) from *Pseudomonas* sp. (25). Although the alignment is not conclusive (23% identity and 69% similarity over a 101 a.a. block, data not shown), it is not unreasonable that *FUN2* may be involved in folate metabolism. Activated forms of tetrahydrofolate are substrates, being metabolised to form tetrahydrofolate, at two points in the purine biosynthesis pathway in which the *ADE1* enzyme functions (26). *FUN2*, then, may play a role in the degradation or salvage of the tetrahydrofolate that is produced, in which case it might be economical and advantageous for *ADE1* and *FUN2* to be co-ordinately regulated. Their close proximity and divergently transcribed mRNAs would allow this.

DISCUSSION

The Tn3 deletion sequencing strategy has some specific advantages:

1. The method is simple to perform, with no *in vitro* manipulations required to make deletions. This may make it particularly attractive to users who do not wish to spend time becoming proficient in the often initially troublesome exonuclease based techniques.

2. Generation of deletions is not labour intensive, even though the procedure takes four days from having the insert cloned into the vector to picking sequencing clones. It should be noted that over these four days, only about four hours of relatively non-technical work is performed (plus the time spent for one transformation of *E. coli*.).

3. The method is inexpensive, requiring only simple media and no costly enzymes to make ordered deletions.

4. The method generates a mutant bank of transposon insertions (in addition to a deletion bank) that can be used for *in vivo* studies involving gene replacement by homologous recombination with chromosomal DNA. The transposon m-Tn3 UFO can be selected for in both *E. coli* and *S. cerevisiae*.

5. The Tn3 deletion strategy may useful as an initial approach to a sequencing project. If initial results are unpromising, the same vectors can be used to make exonuclease III- or T4 polymerase-mediated deletions using currently available strategies, so there is no need to re-clone. The converse is not the case, since most vectors currently in use encode a copy of the Tn3 38bp repeat which prevents further insertions by Tn3. Thus, the Tn3 deletion strategy interfaces well with other strategies.

6. Later in a project, a bias caused by regional specificity of Tn3 insertion may be detected in the population of sequencing clones. If this specificity were to take the form of a severe 'cold spot', it may at some stage become uneconomic to pursue the Tn3 deletion strategy further. In this case it is possible to apply other deletion methods to clones already deleted by transposon mutagenesis. The polylinker in the transposon m-Tn3 FOa can be used to sequence into the cold spot by exonuclease III- or T4 polymerase-mediated deletions, or the Exometh method. Alternatively, a cold spot could be subcloned and re-mutagenised.

7. The Tn3 deletion strategy may be uniquely suited to larger sequencing projects. Exonuclease-based methods can encounter a sequence dependent, 1-dimensional block to digestion during the generation of deletions, which may prevent the subsequent sequencing of a large portion of the insert. The chance of finding one of these blocks increases with the size of the clone, and the amount of DNA unsequenceable beyond it increases exponentially. The strategy described here will probably suffer more from regional transposition 'cold' or 'hot' spots when applied to larger clones, but since the method is not 1-dimensional in nature, the only areas of DNA not sequenced will lie *within* those cold spots and not, in addition, beyond them. More sequence is likely to be obtained from a large fragment, then, even in the face of these artefacts. We have recently sequenced a 9.5 kb fragment using the Tn3 deletion strategy (in preparation).

The strategy outlined here also offers opportunities for future development. The time taken for the Tn3 mutagenesis, for instance, would be reduced by one day if the f1 origin-mediated deletion event were carried out on the intermediate cointegrate molecule. Other strategies may also be developed to lessen the potential effects of site specificity. A single Tn3 38bp terminal repeat remains in the sequencing clone after the deletion event. This could be removed by brief exonuclease treatment from a nearby, unique restriction site (for instance an 8 base pair recognition site on the transposon, close to the terminal repeat). The phagemid would then be susceptible to Tn3 transposition once again. Alternatively, the single-stranded origin deletion strategy may be adapted for use with gamma delta or other transposons, which may then be used to create deletions in clones that have previously been deleted by Tn3. The combined application of these methods may be a powerful approach to sequencing even troublesome fragments.

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