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Integration of the yeast retrovirus-like element Ty3 upstream of a human tRNA gene expressed in yeast

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Abstract

The retrovirus-like element Ty3 of *Saccharomyces cerevisiae* integrates into the yeast genomic DNA in a position specific manner. Ty3 integrates within 1–2 base pairs of the site of transcription initiation by RNA polymerase III. The human tRNA_{3^{lys}} gene was used as a target for transposition in a plasmid-based assay to determine whether Ty3 integration can be targeted to a human tRNA gene. Each transposition event observed was adjacent to the site of initiation of transcription of the human tRNA gene. Therefore, heterologous tRNA genes can serve as targets for Ty3 in yeast. This is a first step toward development of a system for targeted integrations in heterologous organisms. © 1997 Elsevier Science B.V.

Keywords: Transposition; Retrovirus; Retrotransposon

1. Introduction

Integration of viral DNA is an essential step in the life cycle of retroviruses and retrotransposons. Retroviruses can integrate throughout the genome of their hosts. Integration is not random, but the mechanisms that determine the positions of integration are poorly understood (Craigie, 1992; Bushman, 1995). A number of studies indicate that factors influencing the structure of the DNA play a role in target site selection. Assembly of DNA into nucleosomes enhanced integration into the exposed major groove (Pryciak and Varmus, 1992). Bending of the target DNA by DNA binding proteins or at phased tracts of adenosine residues can create favored integration sites in the region where the DNA is distorted (Muller and Varmus, 1994; Pruss et al., 1994b). Integration was targeted preferentially into sites that showed the most severe distortion due to bending of the DNA around nucleosomes (Pruss et al., 1994a). In some examples, interaction between

the integration machinery and DNA binding proteins has been invoked. Integration was stimulated by a specific interaction between HIV IN and a putative transcription activator (Kalpana et al., 1994). Perhaps the most compelling example of preferential insertion is the position-specific integration of the yeast retrovirus-like element Ty3, which is targeted by RNA polymerase III transcription factors (Kirchner et al., 1995).

The yeast retrovirus-like element Ty3 is more closely related to the *Drosophila melanogaster* gypsy-like retroviruses and to animal retroviruses than to the other yeast retrotransposons (Hansen et al., 1988). Ty3 is composed of a 4.7-kb internal domain flanked by 340-bp LTRs (Clark et al., 1988). Ty3 is distinguished from other retrotransposons and from retroviruses by its unique integration specificity. De novo Ty3 integrants isolated from yeast genomic DNA were shown to be located within 1–2 bp of the site of initiation of transcription of tRNA genes (Chalker and Sandmeyer, 1990). Subsequently, it was shown that the RNA polymerase-III-transcribed genes, 5S and U6, can also serve as specific targets for Ty3 integration (Chalker and Sandmeyer, 1992). In addition, the tRNA gene target must be transcriptionally competent since promoter mutations that abolish transcription also prevent integration (Chalker and Sandmeyer, 1992). Experiments utilizing an in vitro integration assay and fractionated transcription extracts showed that transcription factors

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Abbreviations: Ap, ampicillin; bp, base pair(s); *E.*, *Escherichia*; G418, geneticin; kb, kilobase(s); Km, kanamycin; LTR, long terminal repeat; lys, lysine; Nm, neomycin; pol III, RNA polymerase III; tRNA, transfer RNA; 5-FOA, 5-fluoro-orotic acid.

TFIIIB and TFIIC are required for integration, but that RNA polymerase III (pol III) is not required (Kirchner et al., 1995; Connolly and Sandmeyer, 1997).

A concern to those designing retroviral based gene therapy vectors, is the effect of the integrated vector on the expression of genes in the region adjacent to the site of integration. Random integration of a vector could cause aberrant expression of an oncogene or disrupt expression of a tumor suppressor or other essential gene. In addition, the region within which integration occurs can have an effect on the level of expression of the therapeutic vector. In contrast, the yeast retrovirus-like element Ty3 integrates specifically adjacent to tRNA genes without affecting expression of the tRNA gene (Kinsey and Sandmeyer, 1991). Thus, tRNA genes could be a 'safe' site for integration of a therapeutic vector. In addition, tRNA genes are redundant in the human genome and, because they are expressed constitutively, are likely to be located in accessible regions of chromatin in many cell types. Therefore, integration adjacent to a tRNA gene may lead to more predictable levels of expression of the therapeutic vectors than integration into random sites.

We have utilized a plasmid based transposition assay to test whether Ty3 integration can be targeted to a human tRNA gene. This assay enriches for cells in which integration has occurred into the target plasmid, but not for the site of integration. When a target containing the human tRNA_{3^{lys}} gene was used, all integration events recovered were shown to be in the region of transcription initiation, similar to that seen for a yeast tRNA target gene.

2. Experimental and discussion

2.1. Construction of target plasmid containing human tRNA_{3^{lys}} gene

To determine whether a tRNA gene from a heterologous organism could serve as a target for integration of the yeast retrovirus-like element Ty3, the human tRNA_{3^{lys}} gene was cloned into a yeast high-copy plasmid bearing a selectable marker. The construction of the selectable target plasmid bearing the human tRNA_{3^{lys}} gene is described in Fig. 1. Plasmids pSD1448A–C are individual transformants from the same cloning procedure. Each of these identical plasmids was used as a target in the transposition assay.

2.2. Transcription and 5' end mapping of human tRNA_{3^{lys}} gene in yeast in vivo and in vitro

Ty3 integrates within 1–2 bp of the site of initiation of transcription of tRNA genes in yeast (Chalker and Sandmeyer, 1992). In order to determine whether Ty3

would use a human tRNA gene as a target in a similar manner, we needed to determine the position of transcription initiation of the human tRNA_{3^{lys}} gene. The results from this analysis are shown in Fig. 2 and diagrammed in Fig. 3. Primer extension analysis was performed on total cellular RNAs isolated from yeast cells transformed with the htRNA_{3^{lys}} gene (lane 1) and HeLa cells (lane 2) using an oligonucleotide specific for the human tRNA_{3^{lys}}. The major species (indicated by the arrow) corresponds to the +1 nucleotide of the mature tRNA. Primer extension of the human tRNA_{3^{lys}} gene transcribed in vivo in either yeast or HeLa cells yields a similar, although not identical, pattern and the same major RNA species, indicating that the human tRNA_{3^{lys}} gene is transcribed and processed similarly in yeast and human cells. The 5' ends of longer extension products correspond with potential transcription initiation sites at positions -7 and -13 relative to the first coding base of the mature tRNA.

Transcription of a tRNA gene yields a pre-tRNA molecule that is processed at the 5' and 3' ends in order to form the mature tRNA (Geiduschek and Tocchini-Valentini, 1988). To distinguish the 5' ends of the pre-tRNA from the mature tRNA, primer extension analysis was performed on the human tRNA_{3^{lys}} gene transcribed in vitro in yeast pol III transcription extracts (Klekamp and Weil, 1986; Kassavetis et al., 1989). The results of these reactions are shown in Fig. 2, lanes 3–8. Primer extension analysis of in vitro transcribed RNA from the htRNA_{3^{lys}} gene yields a pattern similar to that seen from in vivo transcribed RNA (compare lanes 7 and 1). The addition of heparin to the in vitro transcription reaction prevents the re-initiation of RNA polymerase III on the DNA template, resulting in a single round of transcription and no processing of the pre-tRNA (Kassavetis et al., 1990). Primer extension analysis of the in vitro transcribed htRNA_{3^{lys}} gene in the presence of heparin resulted in products at positions -13, -14, -15, and -16 (lane 8). Pol III typically initiates with purine residues (White, 1994). Potential transcription initiation sites at purines occur at -13, -14, -15, and -16 (refer to Fig. 3 for nucleotide sequence).

The dependence of human tRNA_{3^{lys}} gene transcription on the yeast pol III transcription machinery was shown using a promoter mutation in the htRNA_{3^{lys}} gene. A C56 to G56 mutation in the *box B* promoter element was shown previously to decrease transcription in vitro of the yeast *SUP4* tRNA^{Tyr} gene to 5% of wild type (Allison et al., 1983) by severely reducing the ability of *box B* to bind TFIIC (Baker et al., 1986). In addition, it was shown that a G56 promoter mutation abolishes the ability of a tRNA gene to serve as a target for Ty3 integration in vivo (Chalker and Sandmeyer, 1992) and in vitro (Kirchner et al., 1995). A G56 promoter mutation was introduced into the htRNA_{3^{lys}} gene via single-

Fig. 1. Transposition assay. Yeast strain yDLC221 containing a galactose-inducible Ty3 element was transformed with both a low copy *URA3* donor plasmid (pDLC348) containing a *Nm*^R-marked Ty3 element, Ty3-N, and the high copy *LYS2*, Ap^R plasmid containing the human tRNA^{Lys} gene as a target (pSD1448A–C). The procedure for isolation of integration events into the target plasmid is summarized at the bottom. **Methods:** Construction of the plasmid pDLC348 and the yeast strain yDLC221 were described previously (Chalker and Sandmeyer, 1992). The pSD1448A–C plasmids were constructed by cloning the 414-bp *Hind*III fragment from phtRNA^{Lys} (a generous gift from J. Rosenblatt, U.C. Los Angeles) containing the human tRNA^{Lys} (Roy et al., 1982) gene into the *Hind*III site, which is proximal to the 3' end of the *LYS2* gene on the pDLP6 plasmid (Fleig et al., 1986). A 2.2-kb *Eco*RI fragment from pEGTy3-2 (Hansen et al., 1988) containing the 2- μ m element was then cloned into the *Eco*RI site of the pDLP6 plasmid. The pSD1448A–C plasmids are individual transformants from the same cloning procedure. Each of these identical plasmids was used as a target in the transposition assay. The transposition assay was performed as described previously (Chalker and Sandmeyer, 1992) except for the use of a *LYS2* marker on the target plasmid instead of a *HIS3* marker. Therefore, selective media was deficient in lysine rather than histidine.

Fig. 2. Determination of the 5' ends of human tRNA^{Lys} gene RNAs. Primer extension analysis was performed on RNA isolated from yeast cells transformed with pSD1448 (lane 1), RNA isolated from HeLa cells (lane 2), and from in vitro transcription reactions in yeast transcription extracts using the htRNA^{Lys} gene in the absence (lane 7) and the presence (lane 8) of heparin. The arrow indicates the start of the mature tRNA species (+1). Primer extension analysis was performed on RNA from in vitro transcriptions of the carrier DNA, pIBI-20 (lane 3), the vector backbone pM13KSII+ (lane 4), and the htRNA^{Lys} gene containing a G56 promoter mutation in the absence (lane 5) and the presence (lane 6) of heparin. The sequencing ladder (lanes C, T, A, and G) was generated by sequencing the htRNA^{Lys} gene with the same oligonucleotide used in the primer extension reactions (5'-end-labeled oligonucleotide 357). **Methods:** Yeast strain yTM443 (Menees and Sandmeyer, 1994) was transformed with pSD1448 and grown in synthetic medium lacking lysine. Yeast cells were harvested and total RNA was extracted as described previously (Clark et al., 1988). Cytoplasmic RNA was extracted from HeLa cell monolayers as described previously (Dildine and Semler, 1989). In vitro transcriptions were performed using a yeast extract, BR500, which supports transcription by RNA polymerase III (Klekamp and Weil, 1986; Kassavitis et al., 1989). The reactions contained 110 mM NaCl, 8 mM MgCl₂, 20 mM HEPES pH 7.8, 200 μM ATP, CTP, UTP, 12 μM GTP, 15 μM [α-³²P]GTP (3000 Ci/mmol), 75 fmol template tRNA gene, and pIBI DNA as carrier (for a total of 1 μg DNA/reaction) in a volume of 50 μl. Single-round in vitro transcriptions were performed by pre-incubating the template DNA for 10 min at room temperature with all reaction components, except GTP and [α-³²P]GTP, to form a transcription complex on the DNA. Then, heparin (80 μg/ml), GTP, and [α-³²P]GTP were added, and the reaction was incubated at room temperature for an additional 10 min. Products were extracted with phenol/chloroform and precipitated with ethanol in the presence of 2.5 M NH₄OAc, washed with 70% ethanol, and dried. The products were resuspended in 5 μl TE, and 2 μl was used in the primer extension reactions. Oligonucleotide primer 357, complementary to the htRNA^{Lys} sequences +24 to +51 (5'-CTGGACCCTCAGATTAAGTCTGATGC-3'), was labeled at the 5' end with ³²P using T4 polynucleotide kinase and [γ-³²P]. Approximately 1 ng oligonucleotide was annealed to the template RNA (20 μg for yeast RNA or 40 μg for HeLa RNA) and extended using purified reverse transcriptase (Life Sciences, Inc.) by the method of Carey et al. (1986). Products of primer extension reactions were treated with RNase A and RNase H for 30 min at 37°C to remove (α-³²P)-labeled RNA. Primer extension products were visualized by electrophoresis in 10% polyacrylamide/bis (20:1)/7 M urea gels followed by autoradiography. The DNA sequence ladder was synthesized by annealing the (γ-³²P)-labeled oligonucleotide 357 to htRNA^{Lys} plasmid DNA and extending with Sequenase (U.S. Biochemicals) in the presence of dideoxynucleotides. All reactions were electrophoresed on the same gel; in the figure, lanes 1–6 represent an exposure time of approximately half that of the remaining lanes. The C56 to G56 promoter mutation in the htRNA^{Lys} gene was generated via single-stranded oligonucleotide mutagenesis (Kunkel, 1985) using the oligonucleotide 5'-ACAGGGACTTCACCCCTGGA-3', which is complementary to htRNA^{Lys}. The underlined C nucleotide mutates the C at position 56 to a G in the *box B* promoter element.

stranded oligonucleotide mutagenesis. As expected, in vitro transcription reactions using the G56 mutant template, followed by primer extension analysis, failed to yield a product (lanes 5 and 6). Control extension

reactions performed on in vitro transcription reactions with carrier DNA, pIBI-20 (lane 3) and the vector backbone, pM13IIS+ (lane 4) also did not yield any product.

Fig. 3. Sites of Ty3 integration adjacent to the human tRNA^{Lys} gene. A schematic of the human tRNA^{Lys} gene is shown at the top. The shaded boxes represent the internal promoter elements *box A* and *box B*. The +1 indicates the first nucleotide of the mature tRNA. The nucleotide sequence shown is from -23 to +19 of the human tRNA^{Lys} gene. The triangles above the nucleotide sequence indicate the positions of the 5' proximal (to the tRNA gene) cut made by Ty3 integrase prior to integration. The open and solid triangles indicate the orientations of Ty3 integrations relative to the tRNA gene target. The two orientations are illustrated in the lower right of the figure. The numbers above and below the triangles indicate the number of integration events detected in each of these positions and orientations. The stars followed by arrows indicate the sites of transcription initiation as determined by primer extension analysis of in vivo RNA isolated from yeast cells transformed with the htRNA^{Lys} gene or RNA from the in vitro transcription of the htRNA^{Lys} gene. The 'H' followed by an arrow indicates the site of transcription initiation when heparin is present in the in vitro transcription reaction. **Methods:** Following the assay described in Fig. 1, DNA was isolated from yeast cells which had undergone a transposition event (G418 and 5-FOA resistant) and used to transform *E. coli* to both ampicillin and kanamycin resistance. Plasmid DNA was extracted from *E. coli* transformants (Ausubel et al., 1997). The position of Ty3 integrations was determined by DNA sequence analysis using the method of Sanger et al. (1977), the Sequenase enzyme (U.S. Biochemicals), and oligonucleotide 357, which is complementary to the htRNA^{Lys} gene.

2.3. Ty3 integration adjacent to the human tRNA^{Lys} target

High-level expression of Ty3 RNA results in Ty3 transposition into sites upstream of genomic tRNA genes (Hansen et al., 1988; Chalker and Sandmeyer, 1990). A transposition assay was developed to recover transposed elements in target plasmids independent of the site of integration (Chalker and Sandmeyer, 1992). This assay was used to manipulate the target gene and show that a transcriptionally-competent, RNA polymerase III-transcribed gene was necessary to target integration (Chalker and Sandmeyer, 1992). The transposition assay has been used in this study to determine whether a gene transcribed by pol III, from a heterologous organism can be used as a target for transposition. In this assay, summarized in Fig. 1, the donor Ty3 element, Ty3-N, was tagged with the bacterial Nm^R gene, which confers G418 resistance in yeast and kanamycin resistance in *E. coli*. Transposition experiments were performed using yeast strain yDLC221. This strain

contains an integrated, galactose-inducible, Ty3 helper element that is required to provide the essential proteins in trans because the Ty3 coding region is disrupted by the Nm^R gene. The Ty3-N element was placed on a low-copy, *URA3*-marked plasmid, to produce the donor plasmid pDLC348.

The human tRNA^{Lys} gene was cloned on to a high-copy, *LYS2*-marked plasmid to provide multiple plasmid targets per cell. Three identical copies of the target plasmid were used independently in this assay, pSD1448A–C. Each of the target plasmids was transformed independently along with the donor plasmid pDLC348 into the yeast strain yDLC221. The assay and selection procedure were carried out as described in Fig. 1. For each target plasmid, 12 integration events were analyzed (G418 and 5-FOA resistant). The concentration of G418 (700 µg/ml) used enriched for cells in which Ty3-N insertions had occurred into the high-copy target plasmid. In two out of 36 selected colonies, there was no growth on medium lacking lysine, indicating that these clones may have undergone more than one

chromosomal integration. In order to analyze the integration events into the target DNA, the plasmids were rescued in *E. coli* and selected for growth on ampicillin and kanamycin. From the 34 clones that had undergone plasmid-based integration events, plasmids were rescued from 27 as Ap^R/Km^R colonies in *E. coli*. Restriction enzyme analysis was performed to verify the presence of the Ty3-N element on the rescued plasmids (data not shown). Once the presence of the Ty3-N element was verified, positions of the integration events were determined via sequence analysis of the target plasmids.

In order to determine the exact location of the integration of Ty3, the rescued plasmids were sequenced with an oligonucleotide primer specific to the htRNA₃^{Lys} gene. The sequence was determined for 25 of the rescued plasmids, and the position of Ty3 integration relative to the proposed site(s) of transcription initiation on the htRNA₃^{Lys} gene is shown in Fig. 3. Upon integration, Ty3 makes a 5-bp staggered cut in the target DNA (Clark et al., 1988). The position of the nick closest to the tRNA gene is indicated in the figure. In addition, Ty3 can integrate in either orientation relative to the tRNA gene target, as illustrated in Fig. 3. Integrations were observed in both orientations at equivalent frequencies. The integrations clustered around positions -7 and -13. The 5'-end mapping analysis identified position -13 and the three bases upstream as the primary transcription initiation sites, although products corresponding to position -7 were also observed in both in vitro and in vivo transcription experiments. Therefore, -7 may be a minor initiation site. The majority of integration events (18 out of 25) were targeted to the region around the primary initiation site at nucleotide -13, whereas only six out of 25 integration events were targeted to the putative minor initiation site at -7. All 25 integration events analyzed were targeted to the region of transcription initiation of the human tRNA₃^{Lys} gene.

2.4. Conclusions

We have used the human tRNA₃^{Lys} gene as a target in a plasmid-based transposition assay for integration of the yeast retroviruslike element Ty3. We have shown that the human tRNA₃^{Lys} gene can be transcribed both in vivo and in vitro by yeast factors. This observation is consistent with the consensus promoter elements that are conserved among tRNA genes (Geiduschek and Tocchini-Valentini, 1988; White, 1994). The *box A* and *box B* promoter sequences of the tRNA₃^{Lys} gene only vary from the consensus promoter sequence at two positions. In both cases, the nucleotide present in the tRNA₃^{Lys} gene is the second most frequent nucleotide present at those positions (Pavesi et al., 1994). In light of this conservation of promoter elements, it is not surprising that the yeast transcriptional machinery can recognize and transcribe the human tRNA₃^{Lys} gene.

None the less, we believe this is the first report of a human tRNA gene transcribed in a yeast system.

The human tRNA₃^{Lys} gene served as a target for Ty3 integration in an in vivo assay. Analysis of all recovered integration events revealed that Ty3 integrated adjacent to the site of transcription initiation and in an orientation-independent manner, similar to that seen when using a yeast tRNA gene as a target. This assay is not quantitative, and therefore a direct comparison of the efficiency of integration of the human tRNA₃^{Lys} gene and a yeast tRNA gene cannot be made. Such a comparison is also complicated by the lack of information concerning the relative usage of different yeast tRNA genes as targets for Ty3 transposition. The recently completed genomic sequence of *Saccharomyces cerevisiae* shows that diverse tRNA genes have been used as targets for Ty3 transposition. It is not known whether these genes represent a preferred subset of tRNA gene targets, and if so, what factors influenced the use of these tRNA genes out of the 275 total tRNA genes in the genome (Goffeau et al., 1996). Integration can now be further studied to determine whether human transcription factors can substitute for the yeast transcription factors and target Ty3 integration to a human tRNA gene. This will be an important step in determining whether the position-specific integration properties of Ty3 can be incorporated into a retroviral-based gene therapy vector.

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