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Stabilization of R Loop Structures by Photochemical Crosslinking with 4,5',8-trimethylpsoralen: Application to Gene Enrichment and Molecular Cloning

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Summary: R loops formed between nucleosomal DNA and tRNA can be photochemically crosslinked with 4,5',8-trimethylpsoralen directly in the R loop formation buffer. When biotin is coupled to the tRNA 3'-terminus via a diaminohexan linker and the modified tRNA employed for R loop hybridization the crosslinked R loops can be efficiently purified by affinity chromatography on avidin-glass columns. Following tRNA hydrolysis the partially crosslinked double stranded DNA, highly enriched for tRNA genes can be cloned in E.coli χ1776.

Introduction: When an RNA molecule is hybridized to douplex DNA in the presence of high formamide concentrations and close to the strand dissociation temperature an R loop will be formed by displacement of the identical DNA strand. This reaction has become an useful tool for gene mapping and gene purification (1-4). However, R loops are labil structures which poorly withstand biochemical fractionation and purification procedures. A procedure for the stabilization of R loop structures would therefore be advantageous. The photoreactive furocoumarin, me_3-psoralen (4,5',8-trimethylpsoralen) intercalates into nucleic acids and upon exposure to UV light yields covalent monoadducts and interstrand crosslinks neighbouring pyrimidines (5-10). The photochemical crosslinking of tRNA:DNA R loops, application of this technique to the enrichment of tRNA genes in double stranded nucleosomal DNA, and the subsequent cloning of such DNA is described in the present paper.

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Materials and Methods: 4,5',8-trimethylpsoralen was a gift from Paul B. Elder Company. Total tRNA and $tRNA^{Phe}$ were isolated and purified as described (11). After incubation with alkaline phosphatase they were 5'-end labelled with /^{32}P/ by the T_4-polynucleotide kinase reaction using /γ-^{32}P/ATP (200 Ci/mmol) to a specific activity of 2.5×10^5 cpm/µg (12).

The preparation of biotin-diaminohexan-tRNA (biotin-C_6-tRNA) and the coupling of avidin to aminoalkylsilyl glass was performed as recently described (13,14,15).

/^{32}P/-labelled tDNAs were synthesized as described (16) with the exception that /α-^{32}P/dGTP and /α-^{32}P/dCTP (1000 Ci/mmol) were used as the radioactively labelled dNTPs. The specific activity of the synthesized tDNAs was adjusted to 5×10^7 cpm/µg.

Nucleosomal DNA was isolated from chicken embryo nucleosome di-, tri-, and tetramers and enriched for tRNA genes by RPC-5 chromatography in the presence of a G:C-specific dye (17,18). Cloned nucleosomal DNA containing a $tRNA^{Phe}$ gene was excised from plasmid pBR322N4P2 of our collection of chicken embryo nucleosome tetramer DNA (15). DNA was 5'-end labelled with /^{32}P/ as above. The isolated cloned DNA had to be incubated with alkaline phosphatase prior to labelling to remove 5'-phosphates; non cloned nucleosomal DNA already bears a 5'-OH terminus originating from the staphylococcal nuclease digestion of chromatin (17).

Single-stranded-DNA:tRNA hybridization was performed in 60 µl volumes containing 1 µg/ml cloned nucleosome tetramer DNA, 0.01-0.2 µg/ml /^{32}P/-labelled $tRNA^{Phe}$, 2xSSC, 0.2% SDS, 0.5 mM EDTA. After incubation at 65 °C for 2 h samples were diluted 10-fold with 0.2 M NaCl, 4 mM EDTA, 20 mM Tris-HCl, pH 7.2, digested with RNase A and T_1 (10 µg/ml and 250 U/ml, resp.) at 37 °C for 1 h, and processed for liquid scintillation counting. A background of 0.6% of input counts was substracted.

tDNA hybridization mixtures contained in 60 µl 1-10 µg/ml DNA, 1 µg/ml /^{32}P/-labelled tDNA, 6xSSC, 0.2% SDS, 0.5 mM EDTA. Incubation was at 65 °C for 8 h. Non hybridized tDNA was removed by Sephadex G-50 chromatography. A background of 0.1% of input counts was substracted.

R loop hybridization was assayed in 100 µl volumes containing 1 µg/ml cloned nucleosome tetramer DNA (or 10^4 cpm of /^{32}P/-labelled DNA), 0.01-0.2 µg/ml /^{32}P/-labelled $tRNA^{Phe}$ (or 0.25 µg of biotin-C_6-$tRNA^{Phe}$), 70% formamide, 0.15 M NaCl, 5 mM EDTA, 50 mM triethanolamine, pH 8.0. After incubation at 45 °C for 2 h the samples were allowed to cool slowly to room temperature (2-3 h), diluted 10-fold with 0.15 M NaCl, 2 mM $ZnSO_4$, 50 mM Na-acetate, pH 4.5, and incubated with 40 U/ml of S_1-nuclease at 37 °C for 1 h. The solution was made 5 mM EDTA, neutralized, and incubated with RNase A and T_1 as above.

For the crosslinking of R loops with me_3-psoralen 25 µl aliquots of R loop hybridization assays were diluted with 25 µl of 70 % formamide in H_2O and distributed into 0.2 ml quartz cuvettes. The cuvettes were placed in a cold room (4 °C) 0.5 cm above the filter surface of a long wavelength UV transilluminator (Chromato Vue C/61) with a radiation intensity of 7 mW/cm^2. At 0, 1, 4, and 8 h of irradiation 1 µl of a me_3-psoralen stock solution (0.45 mg/ml in absolute ethanol) was added to each cuvette. The saturation level of me_3-psoralen is 35 µg/ml in 70% formamide. After 12 h of irradiation samples were either precipitated with ethanol and dissolved in 1 mM EDTA, 10 mM Na-

phosphate, pH 7.0 for agarose gel electrophoresis or R loop structures were separated from non hybridized tRNA by Sepharose 4B chromatography.

Samples were denatured if indicated by treatment with 1 M glyoxal in 70% formamide, 1 mM EDTA, 10 mM Na-phosphate, pH 7.0 at 50 °C for 1 h. Agarose gel electrophoresis was performed in a 17.5x22 cm horizontal apparatus. 2% (w/w) gels in 1 mM EDTA, 10 mM Na-phosphate, pH 7.0 were used (19).

For the purification of tRNA genes R loop hybridization was performed in 1 ml volumes containing 1 mg of DNA partially enriched for tRNA genes from nucleosome dimers and trimers (N_2N_3-DNA), 250 µg of biotin-C_6-tRNA prepared from total chicken embryo tRNA, and other ingredients as above. After incubation at 45 °C for 16 h samples were cooled to room temperature by lowering the incubation temperature for 5 °C in 2 h intervalls. Following dilution with 1 volume of 70% formamide 1 ml aliquots were irradiated as above in 3 ml quartz cuvettes. Samples were diluted with 1 ml of 2xSSC, precipitated with ethanol, dissolved in 200 µl of 1 M NaCl, 5 mM EDTA, 50 mM triethanolamine, pH 8.0, and excess biotin-C_6-tRNA was removed by Sepharose 4B chromatography in the same buffer. Biotin-C_6-tRNA:DNA R loops were adsorbed onto a 0.6x5 cm avidin-glass column in the same buffer; the flow was 0.4 ml/min. The column was washed with 20 ml of buffer, heated to 50 °C, and the hybridized DNA was eluted with 99% formamide. Carrier tRNA (50 ug/ml) was added to the eluate, the solution deproteinized,and nucleic acids precipitated with ethanol. tRNA was hydrolized (0.2 M NaOH, 50 °C, 10 h) and the DNA dialysed,after neutralization,against 2xSSC.

The purified crosslinked DNA was inserted into the restriction endonuclease Pst 1 cleavage site of plasmid pBR322 by the G:C joining procedure (20). The recombinant plasmids were cloned in the E.coli strain χ1776 (21). Details of recombination and transformation procedures have been published (15).

Results: Fig.1A shows the amount of chicken embryo $tRNA^{Phe}$ hybridized to cloned nucleosome tetramer DNA containing a $tRNA^{Phe}$ gene under different hybridization conditions. Reassociation between $tRNA^{Phe}$ and single stranded DNA as well as R loop hybridization led with similar $tRNA^{Phe}$ concentrations to the saturation of the $tRNA^{Phe}$ genes present. R loops were assayed according to the observation that the RNA hybridized to double stranded DNA is rendered stable against RNase digestion when the loop of single stranded DNA, displaced by the RNA molecule is first digested with S1-nuclease (1). Approximately 30% of the hybrids formed under the conditions of R loop hybridization are stable against RNase digestion without preceding S1-nuclease treatment

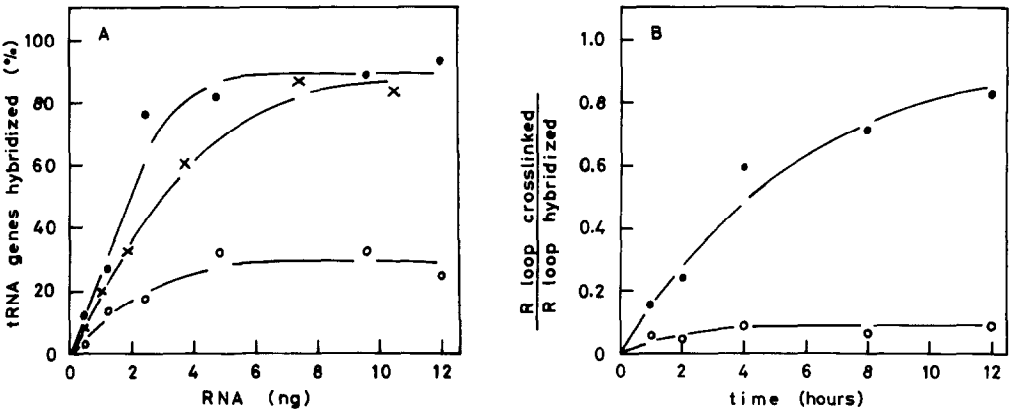

Fig. 1. Hybridization of cloned $tRNA^{Phe}$ gene containing nucleosomal DNA with /^{32}P/-labelled $tRNA^{Phe}$ and photochemical crosslinking of R loops with me$_3$-psoralen.
A (x-x-x) R loop hybridization assayed by S1-nuclease/RNase digestion; (o-o-o) R loop hybridization followed by digestion with RNase; (•-•-•) single-stranded-DNA:tRNA hybridization.
B (•-•-•) photochemical crosslinking of R loops with me$_3$-psoralen and subsequent irreversible denaturation with glyoxal; (o-o-o) me$_3$-psoralen added after R loop hybridization but not irradiated with UV light. Following crosslinking non hybridized /^{32}P/-labelled $tRNA^{Phe}$ was separated by Sepharose 4B chromatography.

(Fig.1A). They are supposed to be hybrids between tRNA and single stranded DNA. Most of the hybrids formed can be stabilized against irreversible denaturation by photochemical crosslinking with me$_3$-psoralen (Fig.1B). When me$_3$-psoralen is added without irradiation only 7% of the hybrids withstand denaturation.

The formation of R loops and subsequent crosslinking can be displayed qualitatively by agarose gel electrophoresis employing nucleosomal DNA hybridized with total tRNA under R loop forming conditions (Fig.2). Formation of the R loop structures brings about a clearly discernible slowdown of the electrophoretic mobility compared to crosslinked but non hybridized nucleosomal DNA (Fig.2A,B). Irreversible denaturation after R loop hybridization and crosslinking additionally shifts the electrophoretic mobility. Obviously, the labelled tRNA is not dissociated from DNA by the glyoxal treatment (Fig.2C,D). No substan-

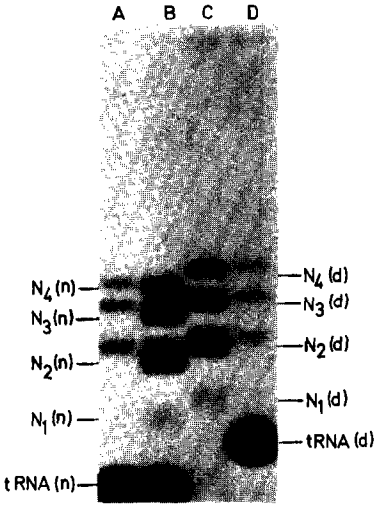

Fig. 2. Agarose gel electrophoresis of photochemically crosslinked R loop structures.
A /^{32}P/-labelled total tRNA incubated under R loop forming conditions with nucleosomal DNA and irradiated with UV light in the presence of me_3-psoralen.
B crosslinked /^{32}P/-labelled nucleosomal DNA together with the same sample as in A.
C crosslinked /^{32}P/-labelled nucleosomal DNA denatured with glyoxal.
D same sample as in A, but denatured with glyoxal.

$N_1(n)$,... $N_4(n)$, and tRNA(n) denote native forms of DNA from nucleosome mono-, di-, tri-, and tetramers, and of tRNA, resp.; $N_1(d)$,... $N_4(d)$, and tRNA(d) refer to the respective glyoxal denatured molecules.

tial amount of labelled tRNA migrates together with the unlabelled DNA when after R loop hybridization me_3-psoralen is added without irradiation (not shown here).

The purification and mapping of genes as RNA:DNA hybrids based on biotin-avidin interaction has recently become an important technique (1-4). However, when applied to double stranded DNA the yields of intact R loop structures are relatively low. We therefore investigated the influence of crosslinking on the binding and recovery of R loops from avidin-glass columns. The cloned nucleosome tetramer DNA containing a $tRNA^{Phe}$ gene was labelled with /^{32}P/, hybridized under R loop forming conditions with biotin-C_6-$tRNA^{Phe}$, and passed over an avidin-

Table 1: Isolation of cloned $tRNA^{Phe}$ genes by affinity chromatography of crosslinked biotin-diaminohexan-$tRNA^{Phe}$:DNA R loops on avidin-glass.

crosslinking ratio* R loop crosslinked / R loop hybridized	DNA bound % of total DNA	DNA recovered % of bound DNA	ds DNA** % of bound DNA
0	8.7 7.6 8.3	12 15 16	5.6 3.5 7.2
0.1	8.5 12 9.2	34 37 35	53 49 55
0.4	26 28 29	51 48 48	67 64 63
0.8	43 47 45	57 53 58	69 65 68

*as obtained from Fig.1B.
** double stranded (ds) DNA assayed as the amount of DNA cleaved by restriction endonuclease Alu I. DNA fragments were separated by agarose gel electrophoresis and the band of undigested [^{32}]-labelled DNA cut out and counted.

glass column. Table 1 shows that binding and recovery clearly depend on the crosslinking ratio. The small amount of DNA bound when R loop samples were not irradiated elutes from the column as single stranded DNA. It probably represents the amount of hybrids between biotin-C_6-tRNA and single stranded DNA always formed under R loop hybridization conditions (see also Fig.1).

The efficiency of tRNA gene purification by the above procedure is demonstrated in Table 2. N_2N_3-DNA partially enriched

Table 2: Purification of tRNA genes by affinity chromatography of crosslinked biotin-diaminohexan-tRNA:DNA R loops on avidin-glass.

yield µg	hybridization cpm hybridized / µg DNA	recovery %**	enrichment factor	total enrichment* factor
4.7	19.05×10^6	43	127	4830
5.8	17.25×10^6	48	115	4370

* Hybridization of DNA extracted from chicken embryo nucleosome di- and trimers (N_2,N_3-DNA) with [^{32}P]-labelled tDNA yields 4.02×10^3 cpm/µg N_2,N_3-DNA. 150 mg N_2,N_3-DNA were partially enriched for tRNA genes by RPC-5 chromatography (18). 70% of total cpm hybridizing in the starting material were recovered 38-fold enriched for tRNA genes (hybridization 0.15×10^6 cpm/µg N_2,N_3-DNA). 1.4 mg each of the partially enriched N_2,N_3-DNA were employed.
** % of total cpm hybridized in DNA 38-fold enriched for tRNA genes.

Table 3: Transformation of E.coli χ1776 with recombinant plasmids bearing crosslinked tRNA gene containing N_2,N_3-DNA.

experiment no.	transformants obtained/1 µg of plasmid DNA		
	tet^r	tet^r/amp^s	hybridizing with /^{32}P/-labelled tDNA
1	121	52	9
2	79	45	4
3	92	53	7
4	117	48	2
5	106	47	5

With 1 µg native pBR322 usually $2x10^5$ transformants were obtained; 1 µg of Pst 1 digested and oligo(dG) extended pBR322 yielded on the average 20 transformants.

for tRNA genes by RPC-5 chromatography was hybridized under R loop forming conditions with biotin-C_6-tRNA prepared from chicken embryo total tRNA. Crosslinking of R loops and subsequent isolation of R loops by affinity chromatography on avidin-glass yields an approximately pure preparation of tRNA gene containing double stranded DNA (18).

The purified double stranded DNA which is partially crosslinked and contains me_3-psoralen monoadducts can be cloned in E.coli χ1776. The number of tet^r/amp^s colonies is only slightly lower than usually obtained in this laboratory with recombinant plasmids bearing non crosslinked DNA (15). However, the number of colonies actually hybridizing with /^{32}P/-labelled tDNA is considerably lower (Table 3).

Discussion: Photochemical crosslinking was used to stabilize tRNA:DNA R loops for tRNA gene purification procedures. Since the small R loops of tRNA molecules cannot be visualized by electron microscopy their formation had to be monitored indirectly by the S1-nuclease/RNase assay. Nevertheless, R loops must have been formed during hybridization and not hybrids be-

tween tRNA and single stranded DNA, since double stranded DNA was obtained in the tRNA gene purification experiments.

The recovery of DNA highly enriched for tRNA genes is 4-5 fold higher with R loops irradiated to a crosslinking ratio of 0.8 compared to the yields obtained with non crosslinked R loops (Table 1). The binding of R loop DNA did not exceed 50% of the total DNA hybridized, probably due to R loops of tRNA not modified with the biotin-diaminohexan-residue. Consequently, purification of biotin-C_6-tRNA from non modified tRNA should further increase the efficiency of the procedure.

The DNA highly enriched for tRNA genes finally obtained contains roughly one me_3-psoralen crosslink per 100 bp of DNA (see Fig.1). According to a recent report (22) the number of me_3-psoralen monoadducts should at least be twice as high. Despite such alteration this DNA can be cloned and bacterial colonies hybridizing with chicken embryo tRNA can be obtained. A comparison of this cloned DNA with tRNA genes of our plasmid collection of chicken embryo nucleosomal DNA will gain information how the bacterial replication machinery deals with the me_3-psoralen modified DNA.

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