ABSTRACT

DNA from chicken embryo nucleosome tetramers (about 760 base pairs in size) was enriched for tRNA genes by RPC-5 chromatography. The enriched DNA was hybridized with chicken embryo total tRNA and the hybridized DNA isolated utilizing a) avidin-biotin interaction, b) diazobenzyloxymethyl paper, and c) high temperature RPC-5 chromatography. The obtained single stranded DNA highly enriched for tRNA complementary sequences was hybridized with total DNA from nucleosome monomers (140-190 base pairs in size) and the excess of non hybridized monomer nucleosome DNA removed by Sepharose 4B chromatography. The hybrid molecules obtained were made fully double stranded by incubation with E. coli DNA polymerase I, DNA ligase, and exonuclease III. DNA was inserted into plasmid pBR322 by G-C joining procedure and the recombinant DNA used to transform the E. coli strain X1776. More than 70% of the transformants obtained hybridize to chicken embryo total tRNA.

INTRODUCTION

Due to the great complexity of the genome of higher eukaryotes difficulties arise when the cloning of a given genome segment by recombinant DNA techniques is intended (1). The purification or at least the enrichment of the genome segment of interest prior to cloning is desirable to increase cloning efficiency. The enzymatic synthesis of double stranded cDNA from purified mRNA has often been used to obtain well defined segments of double stranded DNA (2-10). Although quite useful for a number of applications, cDNAs are not equivalent to eukaryotic genes since they do not reflect regulatory sequences and/or the intron-exon architecture (11-15).

Gene enrichment procedures yielding double stranded DNA make use of restriction enzymes to dissect high molecular
weight DNA. The DNA restriction fragments are fractionated by
density gradient centrifugation (16), gel electrophoresis (17),
RPC-5 chromatography (12), or R loop hybridization and purifi-
cation techniques (18,19). In most cases, however, only a par-
tial enrichment for a given fragment is achieved.

RNA:DNA hybridization allows for the efficient purifica-
tion of RNA complementary DNA if the RNA:DNA hybrids can be se-
parated from excess non hybridized DNA and RNA, respectively
(20,21). Unfortunately, the DNA obtained is single stranded and
per se cannot be cloned by recombinant DNA techniques. In the
present paper we describe the purification of single stranded
nucleosomal DNA highly enriched for tRNA complementary se-
quen ces and subsequent cloning of this DNA in the pBR322/\chi 1776
vector-host system (22).

METHODS

Total tRNA and DNA from nucleosome mono- and tetramers
(N_1^- and N_4^-DNA) were isolated from 13 day old chicken embryos
(23,24). N_4^-DNA was about 50-fold enriched for tRNA genes by
RPC-5 chromatography as described (25).

Isolation of tRNA:DNA hybrids based on avidin-biotin
interaction. a) Preparation of biotin-diaminohexan-tRNA: N-hy-
droxysuccinimide ester of biotin was prepared as described by
Manning et al. (26) and finally crystallized from 2-propanol.
tRNA (10 mg/ml) was oxidized with NaIIO_4 as described by Broker
et al. (27). 1,6-diaminohexan (final concentration 0.8 M) was
coupled at pH 9.0 by incubation at room temperature for 2 h.
Reduction with NaBH_4 was as described (27). tRNA was dissolved
at 20 mg/ml in 0.2 M triethanolamin-HCl, pH 9.0 and NHS-biotin
(30 mg/mg diaminohexan-tRNA) in N,N'-dimethylformamide (final
concentration 90%) was added. The solution was stirred at room
temperature for 2 h, precipitated with ethanol, dissolved and
dialysed against 2xSSC. b) Covalent coupling of avidin to ami-
noalkylsilyl glass: Aminoalkylsilyl glass was prepared from po-
rous glass beads (Corning, CPG 10, 120/200 mesh, 25 nm pore
diameter) with 3-aminopropyltriethoxysilane according to Robin-
son et al. (28). 2 ml of packed aminoalkylsilylglass were acti-
vated with glutaraldehyde and 10 mg of avidin (Sigma GmbH) in 5 ml of 10 mM NaHCO₃ were added. After 3 h at 4 °C the avidin-glass beads were washed extensively with 0.1 M NaCl and stored at 4 °C. c) Hybridization and affinity chromatography: Hybridization mixtures contained in 1 ml: 1 mg/ml N₄-DNA (about 50-fold enriched for tRNA genes)(25), 100 μg/ml biotin-diamino-hexan-tRNA, 80% formamide, 2xSSC. Incubation was at 45 °C for 16 h. Samples were diluted with 1 ml 2xSSC, precipitated with ethanol, dissolved in 200 μl 1 M NaCl, 5 mM EDTA, 50 mM triethanolamin, pH 8.0, and excess biotin-diamino-hexan-tRNA was removed by Sepharose 4B chromatography in the same buffer. Biotin-diamino-hexan-tRNA:DNA hybrids were separated from non-hybridized DNA by passage over a 0.6x5 cm avidin-glass column in 1 M NaCl, 5 mM EDTA, 50 mM triethanolamin, pH 8.0; the flow was 0.2-0.4 ml/min. The column was washed with 20 ml of buffer, heated to 50 °C, and hybridized DNA eluted with 99% formamide. To the eluate carrier tRNA was added. The solution was deproteinized and precipitated with ethanol. tRNA was hydrolyzed in alkali (0.2 M NaOH, 10 h, 50 °C), neutralized, and dialyzed against 2xSSC.

Isolation of tRNA:DNA hybrids using diazobenzyloxymethyl paper (DBM-paper). DBM-paper was prepared from Whatman 540 paper with 1-(m-nitrobenzyloxy)methyl(pyridinium chloride as described by Alwine et al.(29). 12 μg tRNA per cm² of paper were bound. The tRNA-DBM paper was treated with Denhardt's buffer (30) for 24 h. Hybridization was performed in a sealed plastic bag containing: a 1x10 cm strip of tRNA-DBM paper and 2 ml of 0.5 mg/ml N₄-DNA (as above), 50% formamide, 6xSSC, 0.2% each of bovine serum albumine, polyethylene glycol (20000) and polyvinylpyrrolidon. Incubation was at 50 °C for 16 h. The paper strip was washed 3 times in 300 ml of 50% formamide, 6xSSC at 50 °C, placed as a roll into a 30 ml Corex tube containing 5 ml of 99% formamide, and incubated at 80 °C for 15 min with occasional swirling. Hybridized DNA was recovered as above.

Isolation of tRNA:DNA hybrids by high temperature RPC-5 chromatography (31). Hybridization mixtures contained in 1 ml: 1 mg/ml N₄-DNA (as above), 15 μg/ml /¹²⁵I/-labelled tRNA (5x10⁶ cpm/μg), 2xSSC, 0.2% SDS, 0.5 mM EDTA. Incubation was at 65 °C.
for 16 h. The sample was precipitated with ethanol, dissolved in 100 µl of 1.4 M Na-acetate, 0.5 mM EDTA, 10 mM Tris-acetate, pH 7.3, and loaded onto a 85 x 0.4 cm RPC-5 column equilibrated with the same buffer at 70 °C. A non-linear 1.4-1.75 M Na-acetate gradient at 70 °C was employed for elution. Fractions containing tRNA:DNA hybrids were pooled, precipitated with ethanol (carrier tRNA 100 µg/ml), subjected to alkaline hydrolysis, neutralized, and dialysed against 2xSSC.

All analytical hybridizations were performed in 2xSSC, 0.2% SDS, 0.5 mM EDTA at 65 °C. 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 T₁ (10 µg/ml and 250 U/ml, resp.), and processed for liquid scintillation counting (25).

Electron microscope visualization of tRNA:DNA hybrids. Polymethacrylate spheres were prepared by emulsion polymerization (26). The spheres obtained had a diameter of about 30 nm. Avidin was coupled with 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide methyl p-toluene sulfonate (Sigma GmbH). The avidin spheres were purified 2 times by sucrose gradient centrifugation (26) and finally stored in 1M NaCl, 10 mM EDTA, pH 8.5 at 4 °C. Biotin was coupled to iodine oxidized tRNA via a cytochrome c bridge essentially as described by Sodja and Davidson (32). Single stranded N⁺-DNA, as obtained from tRNA:DNA hybrid isolation procedures, was hybridized to biotin-cytochrome-tRNA in 60 µl volumes containing: 100 µg/ml DNA, 100 µg/ml biotin-cytochrome-tRNA, 80% formamide, 2xSSC. Incubation was at 45 °C for 1 h. The sample was dialysed against 1 M NaCl, 5 mM EDTA, 50 mM triethanolamin, pH 8.0, and adjusted to 95 µl. to 30 µl aliquots 1, 10, and 50 µl, resp., of avidin-spheres were added (0.02, 0.2, and 1 mg) and dialysis continued for 24 h at 4 °C.

Samples were spread in 40% formamide, 0.01 M EDTA, 0.1 M Tris-HCl, pH 8.5, 50 µg/ml cytochrome c on a H₂O hypophase (33).

Synthesis of double stranded DNA. 2 µg of single stranded N⁺-DNA, as obtained from tRNA:DNA hybrid isolation procedures, were hybridized with 250 µg of total DNA from nucleosome monomers (N⁺-DNA) in 10 µl of 2xSSC, 0.2% SDS, 0.5 mM EDTA at 65 °C for 16 h. Excess N⁺-DNA was removed by chromatography on a Se-
pharose 4B, equilibrated with 80 mM NaCl, 10 mM NH₄Cl, 6 mM MgCl₂, 1 mM EDTA, 20 mM Tris-HCl, pH 8.1 at room temperature. Peak fractions of DNA hybrid molecules were pooled (80 µl) and incubated in a 100 µl reaction mixture containing: 80 mM NaCl, 10 mM NH₄Cl, 6 mM MgCl₂, 1 mM EDTA, 20 mM Tris-HCl, pH 8.1, 50 µg/ml bovine serum albumine, 0.052 mM NAD, 0.5 mM each of dATP, dTTP, dGTP, and dCTP, 150 U/ml E.coli DNA polymerase I (Boehringer/Mannheim), 15 U/ml E.coli ligase (PL-Biochemicals), and 1 U/ml E.coli exonuclease III (PL-Biochemicals). In analytical studies α-³²P/dCTP (100 µM, 1 Ci/mmol) replaced unlabelled dCTP. Incubation was at 20 °C for 6 h. The mixture was deproteinized and dNTPs removed by "microstep exclusion" i.e. centrifugation through Sephadex LH 60. The excluded volume was extensively dialysed against 140 mM cacodylic acid, 30 mM Tris, adjusted to pH 7.6 with KOH.

Oligo(dC) extension of synthesized double stranded N₄-DNA and oligo(dG) extension of linearized plasmid. Oligo(dC) extension of N₄-DNA: 14 µg/ml synthesized double stranded N₄-DNA, 0.14 mM α-³²P/dCTP (4 Ci/mmol), 140 mM cacodylic acid, 30 mM Tris, pH 7.6 (KOH), 0.1 mM DTT, 1 mM CoCl₂, and 50 U deoxynucleotidyl terminal transferase per µg DNA. Incubation was at 37 °C for 10 min.

Oligo(dG) extension of the linearized pBR322: 100 µg/ml plasmid were digested with PstI (3 U/µg plasmid DNA) in 50 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.0, 100 µg/ml gelatine. Incubation was at 37 °C for 1 h. The solution was deproteinized and extensively dialysed against 0.1 mM EDTA, 140 mM cacodylic acid, 30 mM Tris, pH 7.6 (KOH). 20 µg/ml linearized pBR322 and 0.14 mM dGTP were incubated with terminal transferase as above. Following extension reaction samples were deproteinized and centrifuged through Sephadex LH 60 equilibrated with 0.1 M NaCl, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.4.

Annealing, transformation, colony hybridization, plasmid isolation. 175 ng oligo(dC) extended N₄-DNA and 1 µg oligo(dG) extended pBR322 in 10 µl 0.1 M NaCl, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.4 were heated to 75 °C for 15 min, incubated at 50 °C for 2 h, diluted to 1 ml with the above buffer preheated to 50 °C, and incubated at 50 °C, 40 °C, and 25 °C for 2 h each (39).
For transformation we used the Mn$^{2+}$/Ca$^{2+}$-method of Enea et al.\(^{35}\). Special growth conditions and care during transformation of E.coli \(\chi^{776}\) have been described in detail \((22,34)\). For the identification of tet$^R$/amp$^3$ clones and for colony hybridization the procedure of Beckman et al.\(^{36}\) was used. Plasmid was isolated from total DNA extracts of bacterial cells employing the acid-phenol procedure of Zasloff et al.\(^{37}\). Plasmid DNA was finally purified from low molecular weight contaminants by Sepharose 4B chromatography. For electrophoresis we used 1 or 2% horizontal agarose gels (17.5x22 cm) in 10 mM Na-phosphate, 1 mM EDTA, pH 7.0 \((38)\).

All manipulations were carried out under L2/B2 conditions in accordance with "Richtlinien zum Schutz von Gefahren durch in-vitro neukombinierte Nukleinsäuren (1978)."

**RESULTS**

We have recently described the partial enrichment of tRNA genes in nucleosomal DNA by RPC-5 chromatography employing concentration gradients of salt and G-C specific dye, simultaneously \((25)\). tRNA gene enriched DNA from nucleosome tetramers (\(N_4\)-DNA, about 0.76 kb) obtained by this method was used as the starting material in the procedure described here (Table 1). Assuming that the average length of a tRNA gene is 100 base pairs and only one tRNA gene is present per DNA fragment, 1% of this nucleosomal DNA fragments is estimated to contain tRNA complementary sequences. For the further purification of tRNA complementary sequences by the isolation of DNA from tRNA:DNA hybrids we tested three different methods. Although all the three methods are useful for the present purpose, we have found considerable differences in yield and enrichment (Table 1). Hybridization to DBM-paper bound tRNA results in a good recovery of the hybridizable material but unspecific binding of DNA lowers the enrichment factor. A large excess of E.coli 5S RNA in the hybridization mixture does not improve enrichment. Surprisingly, poly(rA) reduces the background of unspecifically bound DNA (not shown here).

Isolation of hybrids by the avidin-biotin interaction
Table 1. Purification of tRNA complementary N\textsubscript{4}-DNA by tRNA:DNA hybrid isolation

<table>
<thead>
<tr>
<th>DNA</th>
<th>yield ((\mu)g)</th>
<th>hybridization (cpm hybridized (\mu)g DNA)</th>
<th>recovery (%)*</th>
<th>enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>N\textsubscript{4}-DNA**</td>
<td>1000</td>
<td>0.24x10\textsuperscript{5}</td>
<td>63</td>
<td>53</td>
</tr>
<tr>
<td>DNA isolated from tRNA:DNA hybrids:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) avidin-biotin interaction</td>
<td>2.8</td>
<td>21.7x10\textsuperscript{5}</td>
<td>15.6</td>
<td>4690</td>
</tr>
<tr>
<td>b) DBM-paper</td>
<td>15.2</td>
<td>13.7x10\textsuperscript{5}</td>
<td>53.7</td>
<td>2970</td>
</tr>
<tr>
<td>c) high temperature RPC-5</td>
<td>7.3</td>
<td>19.5x10\textsuperscript{5}</td>
<td>36.7</td>
<td>4230</td>
</tr>
</tbody>
</table>

* % of total cpm hybridized in starting material.

** N\textsubscript{4}-DNA enriched for tRNA genes by RPC-5 chromatography has been obtained from two chromatographic runs employing 42 mg each of chicken embryo total N\textsubscript{4}-DNA. Hybridization of total N\textsubscript{4}-DNA with \(^{125}\text{I}\) labelled tRNA yields 4.62x10\textsuperscript{2} cpm/\(\mu\)g DNA. Background hybridization amounts to 1-1.5% of input cpm.

yields a preparation of single stranded DNA more than 90% pure under the above assumptions, but the recovery is low. Since, we had made no attempts to purify biotin-diaminohexan-tRNA from non modified tRNA and DNA, which obviously are not retained on the avidin-glass column (see METHODS and ref. 21,32) probably cause this low recovery. Isolation of hybrids by high temperature RPC-5 chromatography seems to be a compromise concerning enrichment and recovery, and requires minor experimental efforts.

Pooled DNA, as obtained by methods a) and c), rehybridized with biotin-cytochrome-tRNA and labelled with avidin-polymethacrylate spheres for electron microscopy is shown in Fig.1. Although a lot of tangled DNA structures can be observed, sphere labelled tRNA:DNA hybrids can be clearly recognized. Since the labelling efficiency achieved with avidin spheres does not exceed 60% (32), the estimated purity of the observed hybrids corresponds to the hybridization data obtained.

The purified tRNA complementary N\textsubscript{4}-DNA (0.76 kb) was hybridized with a large excess of total DNA from nucleosome mono-
Fig. 1. Electron microscopy of tRNA:N\textsubscript{1} -DNA hybrids. N\textsubscript{1} -DNA highly enriched for tRNA complementary sequences by tRNA:N\textsubscript{4} -DNA hybrid isolation was rehybridized with biotin-cytochrome-tRNA and labelled with avidin-polymethacrylate spheres. Bar (—) represents 1 kb.

mers (0.14–0.19 kb). The latter should be a representative collection of fragments of chicken embryo DNA. Hence, several of these short fragments will associate with the purified N\textsubscript{4} -DNA and form an efficient template-primer system for the synthesis of double stranded DNA. The difference in molecular weight of N\textsubscript{1} -DNA and the N\textsubscript{4} -DNA:(N\textsubscript{1} -DNA)\textsubscript{n} hybrid structure allows for the quantitative removal of excess N\textsubscript{1} -DNA by gel filtration chromatography. Fig. 2A demonstrates that N\textsubscript{4} -DNA:(N\textsubscript{1} -DNA)\textsubscript{n} hybrids have been formed. Upon agarose gel electrophoresis most of the purified tRNA complementary N\textsubscript{4} -DNA (lane III) comigrates with heat denatured, i.e. single stranded, total N\textsubscript{4} -DNA (lane II). The N\textsubscript{4} -DNA is apparently contaminated with a considerable amount of N\textsubscript{3} -DNA. Most of the product from N\textsubscript{4} -DNA/N\textsubscript{1} -DNA hybridization (lane IV) is found in the position of double stranded total N\textsubscript{4} -DNA (lane I). After heat denaturation the hybridization product dissociates into bands of single stranded N\textsubscript{1} -DNA and N\textsubscript{4} -DNA, respectively (lane V).

The N\textsubscript{4} -DNA:(N\textsubscript{1} -DNA)\textsubscript{n} template:primer system was incubated with a mixture of E.coli DNA polymerase I, ligase (NAD), and
Fig. 2. Agarose gel electrophoresis of $N_4^-$-DNA:$(N_1^-\text{DNA})_n$ hybrids and of synthesized double stranded DNA.

(A) Ethidium bromide stained agarose gels. I; reference mixture of 1.25 µg each of $N_1^-$, $N_2^-$, $N_3^-$, and $N_4^-$-DNA. II; as in I but heat denatured. III; 2 µg $N_4^-$-DNA as obtained after tRNA:DNA hybrid isolation. IV; 2.2 µg of $N_4^-$-DNA:$(N_1^-\text{DNA})_n$ hybrid. V; as in IV but heat denatured.

$N_1(n)$...$N_4(n)$ denote non denatured DNA from nucleosome mono-, di-, tri-, and tetramers.

(B) Autoradiographs of agarose gels. Incorporation of $[^{32}\text{P}]\text{dCTP}$ into $N_4^-$-DNA:$(N_1^-\text{DNA})_n$ hybrids by incubation with a mixture of E. coli DNA polymerase I, exonuclease III, and ligase. Aliquots of the reaction mixture were withdrawn at 0.5 h, 1h, 2h, and 6h, resp.

n and d denote non denatured and heat denatured samples, resp.

exonuclease III together with cofactors and deoxynucleosidetriphosphates. The incorporation of $[^{32}\text{P}]\text{dCTP}$ into DNA was monitored by agarose gel electrophoresis and subsequent autoradiography (Fig.2B). After 30 min of incubation non denatured DNA exhibits a broad band of molecules in the size range of $N_4^-$-DNA down to smaller than $N_3^-$-DNA. With increasing time of incubation this band becomes narrowed from both sides, reflecting the hy-
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drolytic as well as the synthetic activities of the DNA poly-
merase/exonuclease mixture. Correspondingly, the bands of de-
natured DNA become smaller with longer incubation but compared
to non denatured DNA they are broader throughout the incuba-
tion. This difference in band breadth indicates that molecules
smaller than the N-DNA template are present even after 6 h of
incubation. Since incubation of the reaction products with S1-
nuclease does not further diminish the band breadth of dena-
tured DNA (not shown here) we argue that incomplete ligation of
neighbouring second strand fragments rather than incomplete
second strand synthesis is the reason for this discrepancy.

The synthesized double stranded DNA was elongated with
oligo(dC) using calf thymus terminal deoxynucleotidyl trans-
ferase and annealed with PstI cleaved oligo(dG) extended plas-
mid pBR322. Annealing conditions were designed to obtain in a
first reaction high yields of G:C joined hybrids of linear
plasmid and N-DNA and to favour the formation of circular re-
combinant plasmid molecules in a subsequent reaction. The re-
combinant plasmid was used to transform the E.coli K12 strain
X1776. Table 2 gives the yields of 3 transformations em-
ploying the same preparation of recombinant plasmid.

Autoradiographs obtained with the colony hybridization
technique of Beckman et al.(36) are shown in Fig.3. 48 tetra-
cycline resistant/ampicillin sensitive clones of transformation

<table>
<thead>
<tr>
<th>experiment</th>
<th>transformants obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>no.</td>
<td>tet&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>149</td>
</tr>
<tr>
<td>2</td>
<td>192</td>
</tr>
<tr>
<td>3</td>
<td>183</td>
</tr>
</tbody>
</table>

Transformants obtained with native pBR322: 176000, 210000, 197000. Transformants obtained with PstI digested and oligo(dG) extended pBR322: 21, 18, 24.
Fig. 3. Colony hybridization of tet^r/amp^g clones as arranged in tissue culture plates (36).
(A) 48 tet^r/amp^g clones of transformation experiment 1 (Table 2) hybridized with chicken embryo total /$^{125}$I/-labelled tRNA.
(B) 36 tet^r/amp^g clones containing tRNA complementary sequences hybridized with 5'-end /$^{32}$P/-labelled tRNA_Lys.
(C) same clones as in (B) but hybridized with 5'-end /$^{32}$P/-labelled tRNA_Lys.
(D) same clones as in (B) but hybridized with 5'-end /$^{32}$P/-labelled 5S RNA (E.coli).

Experiment 1 were incubated under hybridization conditions with chicken embryo /$^{125}$I/-labelled total tRNA (Fig. 3A). 34 clones clearly hybridize to the total tRNA preparation. When 36 clones hybridizing with total tRNA are incubated with chicken embryo 5'-end /$^{32}$P/-labelled tRNA_Lys and tRNA_Lys, respectively, at least 8 clones hybridize with tRNA_Lys (Fig. 3B) and 2 solely with tRNA_Lys (Fig. 3C). The amount of background hybridization is demonstrated with 5'-end /$^{32}$P/-labelled 5S RNA (E.coli) in Fig. 3D. The strong bias to clones carrying tRNA_Lys complement-
tary sequences is a consequence of the initial enrichment of tRNA genes by RPC-5 chromatography (25). The column fractions employed here as the starting DNA material were enriched above the average for tRNA$^\text{Lys}_2$ complementary sequences.

**DISCUSSION**

The DNA from nucleosome tetramers, of which the purification with respect to tRNA complementary sequences and subsequent cloning is described in the present paper, originates from a staphylococcal nuclease digest of chicken embryo chromatin (24). Digestion with staphylococcal nuclease yields DNA fragments bearing 3'-phosphate and 5'-hydroxyl termini, resp. As a consequence enzymes like calf thymus terminal transferase and E.coli DNA polymerase I which require a 3'-OH terminus, or E.coli ligase needing a 5'-terminal phosphate are inactive with such DNA. To overcome this difficulty we employed an enzyme mixture for the synthesis of double stranded DNA which was originally used in Berg's laboratory (40,41) to produce covalently circular DNA. In this mixture E.coli exonuclease III removes the 3'-terminal phosphate groups, thus preparing a DNA substrate for the synthetic activity and 3'-exonuclease activity of DNA polymerase (42) as well as for the oligo(dC) extension by terminal transferase. The double strand specific 5'-exonuclease activity of DNA polymerase converts 5'-terminal hydroxyl groups to the 5'-phosphates (43) required by E.coli ligase in the enzyme mixture. Obviously, neither DNA polymerase I alone (4-7) nor the Klenow fragment of DNA polymerase I (44) lacking the 5'-exonuclease activity could be used for the present purpose.

With the method described purified single stranded nucleosomal DNA is quantitatively converted to double stranded DNA, which can be used to construct a recombinant plasmid. The high yields of transformed clones containing tRNA complementary sequences could imply that during purification of N$_4$-DNA by tRNA:DNA hybridization little or no DNA:DNA association occurs. Cloning of tRNA identical DNA strands (obtained from DNA:DNA association) would necessarily decrease the yield of tRNA comp-
plementary sequences in the transformed colonies. However, the presence of tRNA genes on both strands of the original DNA from nucleosome tetramers would lead to similar high yields. We assume that the rationale of this purification and cloning procedure, i.e. purification of a long single stranded DNA molecule, reassociation with short non purified total DNA fragments, removal of non hybridized short fragments, and subsequent synthesis of a double stranded DNA, will also apply to the cloning of a given sequence in DNA fragmented by restriction endonucleases.

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