Characterization of a cloned ribosomal fragment from mouse which contains the 18S coding region and adjacent spacer sequences

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ABSTRACT

The large EcoRI fragment of mouse ribosomal genes containing parts of the non-transcribed spacer, the external transcribed spacer located at the 5' end of the precursor molecule and about two thirds of the 18S sequence has been cloned in bacteriophage λ gtWES. A physical map of the DNA was constructed by cleavage with several restriction endonucleases and hybridization of the restriction fragments of the recombinant DNA with labelled 18S and 45S rRNA. The orientation of the inserted fragment as well as the length of the 18S sequence was determined by electron microscopy of R-loop containing molecules. The absence of hybridization of the cloned fragment to other fragments in the genome shows that the non-transcribed spacer does not have a significant length of sequences in common with other sequences in the genome.

INTRODUCTION

The ribosomal genes in eukaryotes occur in clusters of tandemly repeated units. Our knowledge about the organization of rDNA in higher vertebrates has mainly been derived from studies on Xenopus where the ribosomal genes are several thousand-fold amplified. The reiteration frequency of the ribosomal genes in other vertebrates is usually much less and therefore the organization of the repeating units is not well elucidated. The approximately 100 copies of rRNA genes of mouse (1) are organized in very large units at least 44.000 base pairs long (2), each of which contains spacer DNA in addition to the structural genes. EcoRI recognizes 2 - 3 cleavage sites in the non-transcribed spacer region and two in the codogenic region. Thus digestion of mouse rDNA results in two fragments which hybridize to 28S and/or 18S rRNA: (I) a 6.7 kb fragment which contains about one third of the 18S sequence, the internal transcribed

spacer and most of the 28S coding region and (II) a 13 - 15 kb
fragment which contains parts of the non-transcribed spacer, the
transcribed spacer at the 5' end of the 45S pre-rRNA molecule
and two thirds of the 18S sequence (see Fig.2). The shorter
fragment has recently been cloned and characterized (3) and will
prove a useful tool for studies on the maturation process of 18S
and 28S rRNA involving the analysis and sequencing of the target
sites of eukaryotic rRNA processing enzymes. Studies on the ini-
tiation process and the control of transcription of the riboso-
mal genes, however, require cloning of the fragment containing
the 5' end of the precursor molecule as well as segments pre-
ceding the transcribed sequence. In this paper molecular cloning
of the large EcoRI fragment of mouse rRNA in bacteriophage λ
is reported. The inserted sequence has been characterized by
restriction enzyme cleavage, hybridization analysis and the
R-loop technique. The availability of the cloned fragment and
purified chromosomal proteins should make it possible to esta-
blish an in vitro system for the transcription of ribosomal
genes and to analyze influencing factors.

MATERIALS AND METHODS

Construction and Propagation of the Recombinant Phage λ gtWES
Mr 974

The fractionation of mouse DNA as well as the molecular cloning
of the large EcoRI fragment of mouse in bacteriophage λgtWES
was performed in P. Leder's laboratory at the NIH. EcoRI cleav-
ed DNA from a mouse plasmacytoma, MOPC-149, was fractionated
by RPC-5 chromatography (3). The second peak which hybridized
to a mixture of 18 and 28S RNA was pooled and fractionated by
size on a preparative electrophoresis device (4). Fractions
with a mean size of 14 kb were pooled, phenol extracted, etha-
nol precipitated, and ligated to the outer arms of the EK2 vec-
tor λgtWES · λ B (5) and transfected into E.coli LE392.

Recombinants containing ribosomal gene sequences were identi-
fied using the plaque hybridisation technique described by Ben-
ton and Davis (6) using iodinated sea urchin rRNA as a probe.
Positive clones were amplified by transferring the plaques with

a Pasteur pipette to 2 ml Luria broth containing one drop of a
fresh overnight culture of E.coli LE392. The suspension was in-
cubated with vigorous shaking at 38 - 39°C to lysis (6 - 15
hours). Two drops of chloroform were added and the lysate sha-
ken for an additional 5 min. Debris was removed by centrifuga-
tion for 10 min. at 6,000 xg. To the supernatant $MgSO_4$ was added
to 10 mM and gelatine to 0.1%. Usually titers of 1 - 1.5 x 10^{10}
phages/ml were obtained.

For propagative growth bacteria were cultured until a density
of A_{575} = 0.3 (2 x 10^8 cells/ml) was reached. After addition
of 10 mM $MgSO_4$ the bacteria were infected with phages at a
m.o.i. of 1. The culture was shaken slowly for 5 - 10 min to
allow adsorption of the phages and then vigorously shaken un-
til lysis (approximately 4 h). The phages were precipitated
from the cleared lysate by 10% polyethylene glycol (PEG 6000)
in the presence of 0.5 M NaCl. After standing for 2 - 15 hours
at 0°C the phages were collected by centrifugation and resus-
pended in 20 mM Tris-HCl, pH 7.5; 10 mM $MgSO_4$.

Residual PEG was removed by extraction with chloroform and the
phages purified by equilibrium centrifugation in CsCl.

DNA and RNA Preparation

To prepare rRNA Ehrlich ascites cells were extracted with 0.5%
sodium naphthalene-1,5-disulfonate, a method described by Kirby
(7) to selectively extract cytoplasmic RNA. After deproteinisa-
tion the RNA was fractionated by two centrifugations at 5°C in
an SW41 rotor for 15 hours at 27.000 rpm on 15 - 30% sucrose
gradients in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA,
0.2% sodium dodecylsulfate (SDS), 0.1% sodium deoxycholate. The
pooled 18 and 28S RNA was radioiodinated by a procedure modified
from that of Commerford (8). 2 μg of RNA were ethanol precipi-
tated and dissolved in 6 μl 1.5 M Na-acetate, pH 4.5, 3 μl 0.1 M
KJ and 12 μl 50 mM $TlCl_3$. This solution was mixed within a hood
with 3 mCi sodium iodide (10 Ci/15 μl) which had been neutra-
lized with 0.3 N H_2SO_4/0.1 N Na_2SO_3. Iodination was carried out
in a siliconized sealed capillary for 30 min at 65°C. The ca-
pillary was then chilled and its content was expelled into 50 μl

7 mM Na_2SO_3. After extraction with 200 µl chloroform to remove
free iodine the sample was incubated for 10 min at $70^{o}C$ and then
passed through a Sephadex G-50 column in 10 mM Tris-HCl, pH 7.5,
10 mM NaCl, 10 mM EDTA, 0.1% SDS. The ^{125}J-labelled RNA which e-
luted in the void volume was ethanol precipitated in the pre-
sence of 200 µg/ml E.coli tRNA. The specific activity of the
RNA was 1 - 2 x 10^{8} cpm/µg.

High molecular DNA from mouse cells was extracted according to
Gross-Bellard et al.(9). To prepare recombinant DNA phages were
purified by equilibrium centrifugation in CsCl and dialyzed a-
gainst 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA. They
were treated with proteinase K (2 mg/ml) for 30 min at $37^{o}C$ fol-
lowed by subsequent phenol and chloroform extractions.

The DNA was dialyzed extensively against 20 mM Tris-HCl,pH 8.0,
0.1 mM EDTA before used for restriction analysis. Nick-transla-
tion of the DNA with ^{32}P-dGTP was performed according to Mania-
tis et al. (10).

Restriction Enzyme Digestion and Gel Electrophoresis

Restriction enzyme digestions were routinely carried out for 1
hour in the assay recommended by the suppliers. Commercial en-
zymes were purchased both from Boehringer (EcoRI, BamHI, PstI,
HindII) and New England Biolabs (KpnI, XhoI, SalI, SacI). BglII
and XbaI were kindly donated by R.E. Streeck (Institut für
Physiologische Chemie, Universität München) and BalI was a gift
from E.L. Winnacker (Institut für Biochemie, Universität Mün-
chen).

DNA fragments were run on o.7% or 1% vertical agarose gels for
4 1/2 hours at 75 mA in 5o mM Tris-acetate, pH 8.o5, 2o mM Na-
acetate,18 mM NaCl, 2 mM EDTA, o.5 µg/ml ethidium bromide. The
gels were calibrated by running in parallel with the restricted
DNA the following marker digests: -HindIII, -EcoRI, T7-HpaI. The
number of base pairs of the restricted DNA fragments was deter-
mined graphically from a semi-logarithmical plot of these stan-
dard DNA fragments sized in kilobases (kb) versus relative mobil-
ity in cm. The length of the standard DNA fragments was taken
from Philippson et al. (11) and McDonell et al. (12).

For the isolation of specific fragments the restricted phage DNA
was separated on preparative disc gels. Appropriate bands were
cut out and the DNA fragments were eluted from the gel slices by
electrophoresis. The DNA was further purified by chromatography
on benzoylated DEAE-cellulose and concentrated by ethanol pre-
cipitation. After adsorption and washing in 1oo mM NaCl, 1o mM
Tris-HCl, pH 7.4 the DNA was eluted with 1 M NaCl, 6 M urea,
1o mM Tris-HCl, pH 7.4 and concentrated by ethanol precipitation.

Identification of Restriction Enzyme Fragments Containing rRNA
Genes

Elutions from agarose gels into nitrocellulose filters were per-
formed by the method of Southern (13). DNA-RNA hybridisations
were done in sealed plastic bags under the following conditions:
2 x SSC, 1 mM EDTA, o.1% SDS, 68°C, 2o hours. The concentration
of ^{125}J-labelled 18S and/or 28S RNA (spec. act. 10^8 cpm/µg) was
o.o1 µg/ml; an excess of unlabelled E.coli tRNA was present at
2oo µg/ml. If a ^{32}P-nick-translated DNA probe was used the
filters were pre-incubated for 6 hours at 42°C in hybridization
solution in the presence of 1 mg/ml T7 DNA and then hybridized
for 2o hours at 42°C in 5o% formamide, 5 x SSC, o.1% SDS, o.2 mM
EDTA, o.o2% each of ficoll, bovine serum albumin, and polyvinyl-
pyrrolidone (14). After washing for several hours in the buffers
used for hybridisation the filters were dried and autoradio-
graphed at $-7o^{\circ}$C.

Preparation and Visualization of R-loop Containing Molecules

1 µg of mouse hybrid phage DNA was incubated with 3 µg of 18S
RNA and/or 5 µg 28S RNA in 50 µl 70% formamide, 0.3 M NaCl,
10 mM Tris-HCl, pH 8.5, 1 mM EDTA for 3 hours at 64°C as des-
cribed by Wellauer and Dawid (15). The sample was then diluted
20-fold in 50% formamide, 100 mM Tris-HCl, pH 8.5, 10 mM EDTA,
50 µg/ml cytochrome C and spread onto a distilled water hypo-
phase. Samples were picked up with parlodion-coated grids,
stained with 90% uranylacetate in 90% ethanol, washed with 90%
ethanol, and rotary shadowed with platinium-palladium. The sam-
ples were visualized in an electron microscope Elmiskop 102
(Siemens) at a magnification of 8000 : 1. The length of the mo-

lecules and the looped structures was measured and referred to kilobase units by comparison to PM2 DNA.

Containment Conditions

The construction of the hybrid phages and all experiments with
λ gtWES Mr974 and analogous clones were carried out under L3/B2 conditions as specified by "Richtlinien zum Schutz vor Gefahren durch in vitro rekombinierte Nukleinsäuren" of the BMFT of the Federal Republic of Germany. The safety containments are si-milar to the conditions of P3 + EK2 containment required for these experiments by the NIH Guidelines for Recombinant DNA Re-search in the U.S.A. The recombinant clone λ gtWES Mr100 was pro-pagated under L2/B2 conditions since this clone has been lowered to P2 + EK2 containment conditions by the NIH Biohazard Commit-tee.

RESULTS

Cloning of the Ribosomal Fragment of Mouse in λ gtWES: λ B

The restriction endonuclease EcoRI cleaves mouse ribosomal DNA in the region of the so-called non-transcribed spacer, once in the 18S and once in the 28S sequence, yielding two fragments which hybridize to labelled ribosomal RNA (Fig. 1) : a 6.7 kb fragment which contains the 18S and 28S RNA sequences separated by the internal spacer, and a larger fragment about 13 -15 kb in
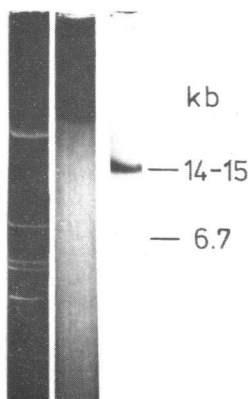

kb

— 14-15

— 6.7

Fig.1 Detection of restriction frag-ments of mouse genomic DNA containing rRNA sequences. 10 µg of high molecular mouse DNA were restricted with EcoRI, concentrated by ethanol precipitation and fractionated by electrophoresis on a 0.7% agarose gel. After transfer to Millipore filters ribosomal sequences were detected with radioiodinated 18S and 28S rRNA.
A + B) Ethidium bromide stained gel showing the position of a λ EcoRI marker digest (A) and the restricted mouse DNA (B).
C) Autoradiograph of ^{125}J-18S and 28S rRNA hybridisation.

length containing two thirds of the coding sequences for the 18S
RNA together with the transcribed spacer located before the 18S
region, and parts of the non-transcribed spacer. A third fragment
described by Cory and Adams (2) which contains the 3'-end of the
28S gene and parts of the non-transcribed spacer could not be
identified by us.

The construction of the hybrid phage λgtWES Mr100 containing the
6.7 kb fragment of mouse rDNA has been described recently (3).
The phage has been kindly donated to us by P. Leder. The purifi-
cation and the cloning of the 13 - 14 kb fragment followed a si-
milar protocol. Actually, the mouse rDNA sequence were isolated
from a pool of clones originally generated under conditions op-
timized for the 14 kb mouse β-globin EcoRI fragment (16). The
rationale of searching within the β-globin optimized pool was
that both the β-globin EcoRI fragment and the large ribosomal
fragment eluted at the same position by RPC-5 chromatography
and are similar in size. Therefore, the DNA fraction used to
construct the β-globin clones still contains in addition to many
other sequences the ribosomal fragment.

Because of the high reiteration frequency of the ribosomal genes
a surprisingly high percentage (about 9%) of clones containing
ribosomal sequences was found. Positive clones were plaque-puri-
fied and preparatively grown for further characterization. Here
we present an analysis of only one of these clones - called
λgtWES Mr974. In Fig. 2 the location of the EcoRI cleavage
sites within mouse rDNA as well as the designation of hybrid
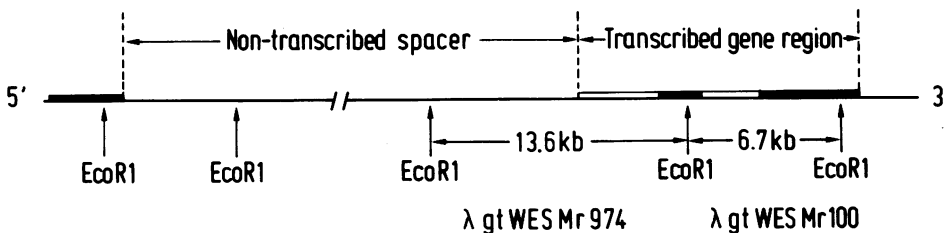phages containing both fragments of the ribosomal genes is re-

Fig. 2. Location of EcoRI cleavage sites within mouse rDNA and
the designation of the hybrid phages which contain either frag-
ment coding for rRNA. The dark area within the transcribed gene
region represents the 18S and 28S rRNA sequences, respectively.

presented.

Length Determination and Sequence Specificity of the Inserted rDNA Fragment

Agarose electrophoresis of DNA from the hybrid phages λ gtWES Mr100 and 974 cleaved with EcoRI endonuclease gave the expected pattern for a λgt hybrid (Fig. 3). Three bands are visible representing the right (13.9 kb) and the left arm (21.3 kb) of λ and the inserted DNA fragment. The length of the insert is 6.7 kb for λ gtWES Mr100 and 11.35 kb for λ gtWES Mr974, respectively. Both phage DNAs hybridize with radioiodinated 45S pre-rRNA and 18S RNA (Table 1). Whereas Mr100 shows strong hybridization with 28S rRNA, Mr974 DNA does not contain 28S sequences. The weak hybridisation with ^{125}J-28S RNA may be competed by cold 18S rRNA, so we attribute it to traces of 18S RNA in the 28S RNA probe.

To prove more directly that the hybrid phage contains only the 5′ end of the pre-rRNA the so-called Northern blotting technique described by Alwine et al. (17) was employed. A mixture of 18S and 28S rRNA was separated by agarose gel electrophoresis, covalently coupled to diazobenzyloxymethyl-cellulose paper and hybridized to ^{32}P-labelled DNA of phage λgtWES Mr1oo and Mr974,
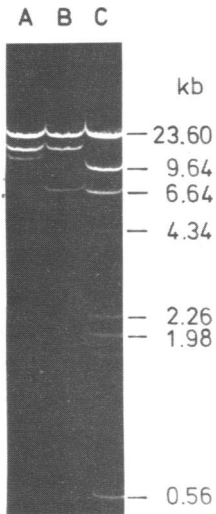

kb
— 23.60
— 9.64
— 6.64
— 4.34
— 2.26
— 1.98
— 0.56

Fig. 3 Cleavage of λgtWES Mr1oo and Mr974 DNA by EcoRI. o.5 µg of DNA isolated from the hybrid phages was digested with EcoRI and subjected to electrophoresis on a o.7% agarose gel.

A) λ gtWES Mr974
B) λ gtWES Mr1oo
C) λ HindIII marker

Table 1 Hybridization of 18S, 28S and 45S rRNA to DNA from the Hybrid Phages λgtWES Mr1oo and Mr974.

Hybridization Probe	DNA from Phage	
	Mr100	Mr974
^{125}J-18S RNA	8.98o	14.87o
^{125}J-28S RNA	37.37o	1.29o
^{125}J-45S RNA	53.2oo	43.95o
+ cold 18+28S rRNA (25µg/ml)	13.87o	33.58o

1µg phage DNA was loaded onto Millipore filters and hybridized in a total volume of 25oµl with radioiodinated RNA (5 x 1o^5 cpm).

respectively. As shown in Fig. 4 the recombinant phage Mr1oo which contains the shorter fragment hybridizes to both 18S and 28S RNA, whereas Mr974 hybridizes exclusively to the 18S band.
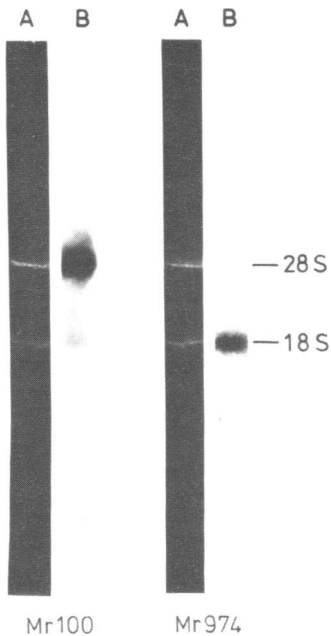

A B   A B

—28S

—18S

Mr100   Mr974

Fig. 4 Hybridization of ^{32}P-labelled DNA of Phage λgtWES Mr100 and Mr974 to Ribosomal RNA coupled to Diazobenzyloxymethyl Paper.

1 µg of each 18S and 28S RNA was separated on a 1% agarose gel in 9o mM Tris-borate, pH 8.3, 2.5 mM EDTA, transferred to diazobenzyloxymethyl paper as described by Alwine et al. (17) and hybridized to nick-translated phage DNA.

A) Ethidium bromide stain of 18S and 28S RNA
B) Hybridization of labelled recombinant DNA to rRNA

A Restriction Map of the Large EcoRI Fragment of Mouse rDNA

The availability of the hybrid phage λgtWES Mr974 makes it pos-
sible to map regions of the ribosomal genes located downstream
the 18S sequence. Fig. 5a shows the fragments which are generat-
ed by SalI digestion in the presence and absence of EcoRI. SalI
digestion alone results in 7 fragments: 23.25, 12.7, 4.6, 3.3,
1.75, o.57 and 0.48 kb in length. Double digestion of λgtWES
Mr974 with SalI and EcoRI generates 9 fragments: 21.3 kb (the
left arm of λ), 12.7 kb (from the right end of λ) as well as
5 fragments 3.8, 3.3, 1.95, 1.75 and 0.57 kb which originate
from the inserted DNA segment. The small fragment o.48 kb comes
from the two adjacent SalI sites in the right arm of λ. The
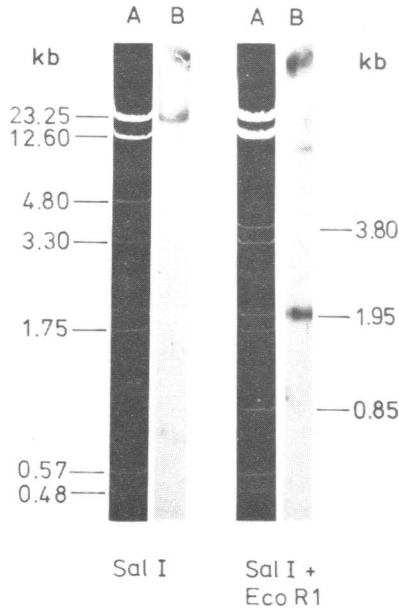0.81 kb fragment indicates the distance of the EcoRI cleavage

Fig. 5a Fragments Detected in Digests of λgtWES Mr974 DNA Made
with SalI and SalI plus EcoRI 1.8 μg of λgtWES Mr974 DNA were
digested with SalI. After 30 min one half of the incubation
mixture was made 100 mM in NaCl and digested for further 30 min
with EcoRI.

A) Ethidium bromide stain of fragments
B) Autoradiograph showing hybridization of [125]J-18S RNA to the
   fragments

site to the first SalI cleavage site on the right arm.

The [125]J-18S probe hybridizes to the largest fragment in the SalI
digest and to the 1.95 kb fragment in the double digest, respec-
tively. This places the 18S coding sequences adjacent to the left
arm of λ (see Fig. 5b).

For more detailed mapping the inserted fragment was isolated by
preparative electrophoresis, electroelution, and subsequent puri-
fication on a benzoylated DEAE column. The purified fragment was
digested with several restriction enzymes. The position of a re-
striction cut in the DNA was determined both by double digestion
with two enzymes or by partial digestion with one enzyme followed
by hybridisation of the fragments to iodinated 18S $_{or}$ 45S RNA,
respectively. The location of the restriction sites is shown dia-
gramatically in Fig. 6. One remarkable feature of this map are
the closely spaced SmaI and PvuII sites in that part of the non-
transcribed spacer which preceeds the start of the transcription
unit. In this region a high variability between different clones
was observed (not shown here). Furthermore, it is noteworthy that
the restriction nucleases -tested so far - preferentially cut the
right part of the rDNA fragment which contains the codogenic se-
quences. This suggests that the average base composition of the
non-transcribed spacer differs from that of the coding region.
The sum of the base pairs of the restricted fragments allows a
rather accurate determination of the length of the inserted seg-
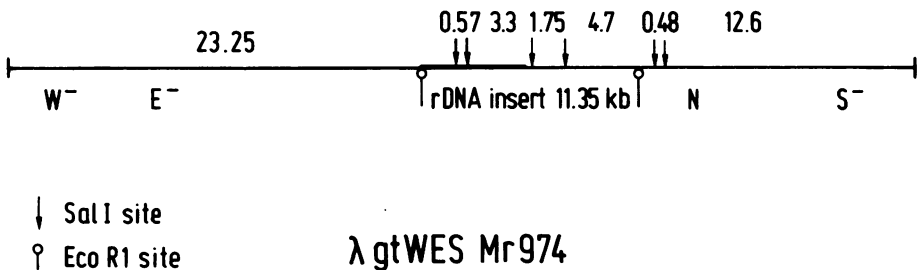ment, that is 11.35 kb. This is slightly smaller than the length

0.57 3.3 1.75 4.7 0.48 12.6

23.25

W⁻   E⁻   rDNA insert 11.35 kb   N   S⁻

↓ SalI site
ᵠ Eco R1 site λ gtWES Mr 974

Fig. 5b Diagramatic representation of the orientation of the ri-
bosomal fragment inserted in λgtWES and the position of SalI en-
donuclease cleavage sites. The darker line indicates the sequen-
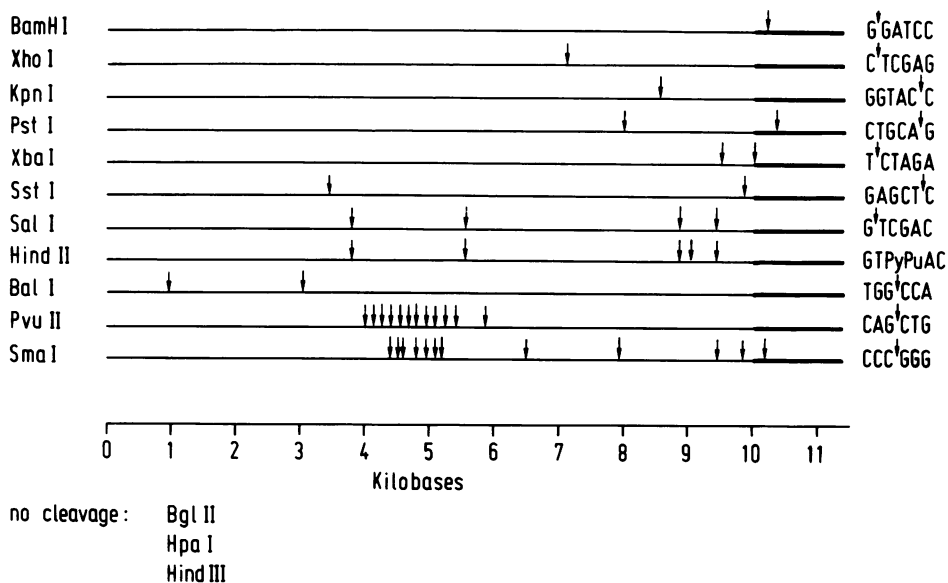ce of 45S pre-rRNA.

Fig. 6 Physical Map of Restriction Enzyme Sites in the Inserted
        rDNA Fragment of λgtWES Mr974

The arrows indicate the location of cleavage sites within the
insert. The darker line ait the right side of the fragment re-
presents the position of the 18S sequence.

of the fragments (about 14 kb) used for ligation and transfection.

Visualisation of the Ribosomal Sequences within the Cloned DNA

In order to establish the orientation of the insert and to esti-
mate the length of sequences encoding ribosomal RNA in the hybrid
phages R-loops were formed by incubating recombinant phage DNA
with ribosomal RNA. Fig. 7A illustrates one typical DNA molecule
of phage Mr1oo which had been incubated with a mixture of 18S and
28S rRNA. A small and a big loop are visible which are separated
by a long stretch of double stranded DNA. This configuration re-
presents the sequences of 18S and 28S RNA as well as the region
of the internal transcribed spacer. The long DNA strands at both
sides of the loops are the long (left) and the short (right) arm
of   between which the foreign DNA had been inserted.
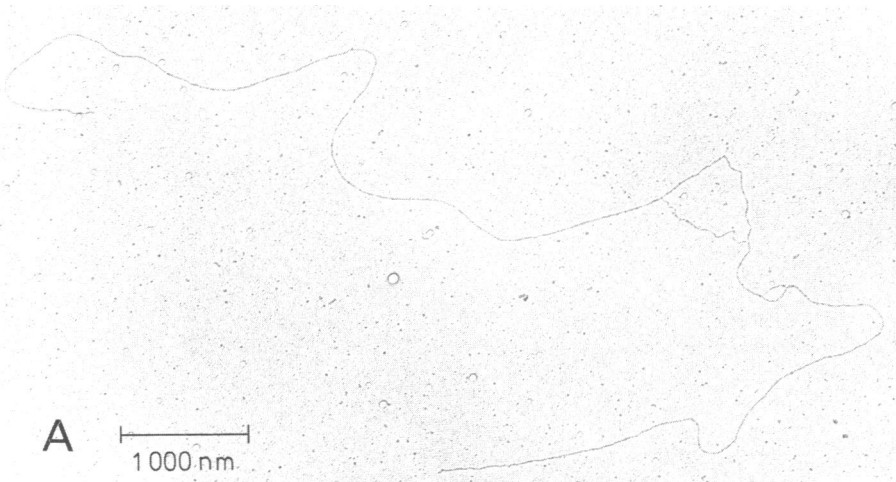If DNA from the hybrid phage Mr974 or analogous clones was ana-

Fig. 7A   R-loops formed by gtWES Mr1oo DNA and 18S + 28S rRNA
          The conditions for annealing and electron microscopy
          are indicated in Materials and Methods.

lyzed only one loop was visible which indicates the position of
the 18S sequence in the inserted fragment (Fig. 7B and C). The
average length of the bubble is 3% of the hybrid phage DNA -
this is 1.4 kb. A similar number (1.38 kb) was obtained by com-
paring the length of the looped structure with PM2 DNA (9.7 kb)
which served as an internal length marker. The size of the loop
was identical in several clones analyzed all of which contained
the large EcoRI fragment from mouse but differed slightly in the
length of the fragment and in the orientation of the insertion.
The orientation of the inserted fragment within the phage λ gtWES
can be easily determined by the R-loop technique as well. The re-
lative distance of the 18S region to the two λ arms indicates
that in λ gtWES Mr974 the 18S sequences are adjacent to the
long left arm of λ . Therefore the orientation of the ribosomal
gene in this hybrid phage is such that the "sense"strand of the
inserted fragment should be under control of the leftward promo-
tor of λ . For comparison, a molecule of another clone - called
λ gtWES Mr 969 - which contains the same inserted sequence in
the opposite direction is shown (Fig. 7C). In this case the 18S
loop is adjacent to the shorter arm of λ . The orientation of
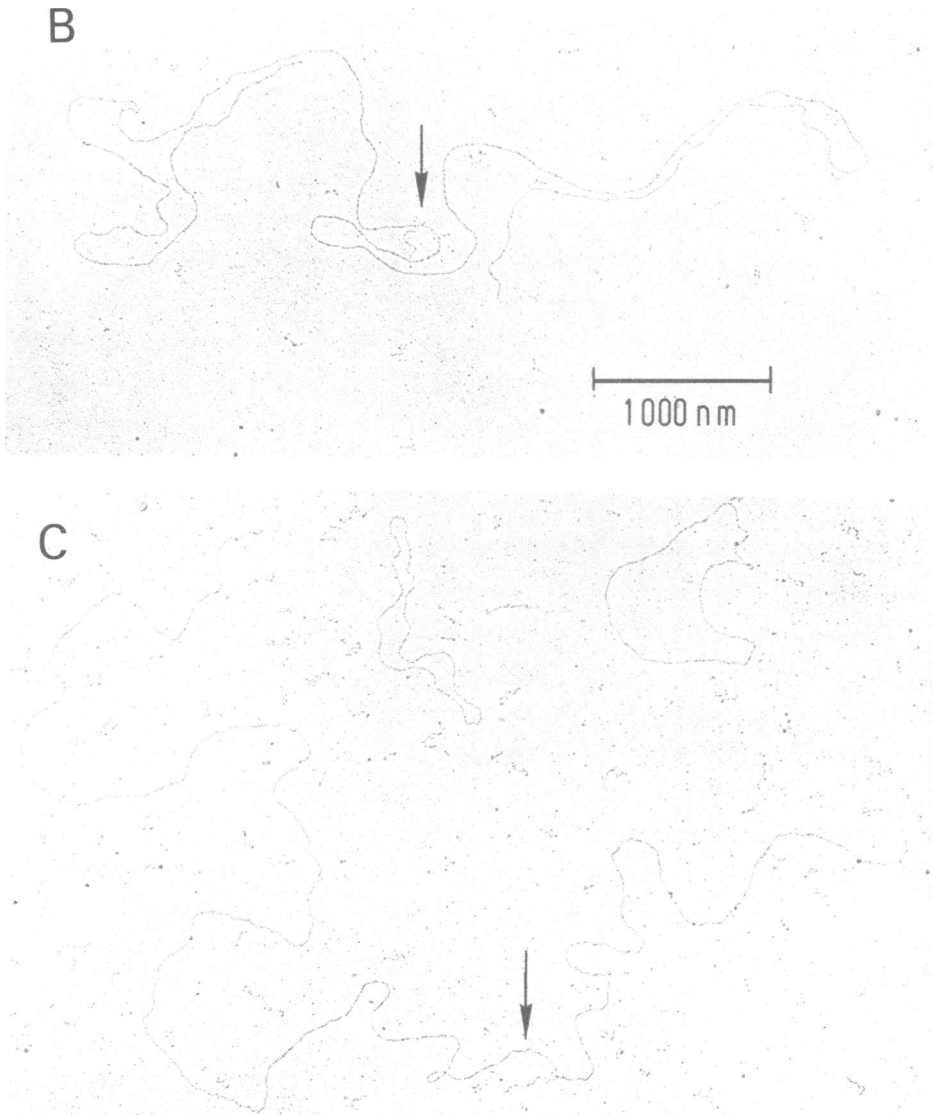
B

1000 nm

C

Fig. 7B and C) 18S RNA R-loop structures in two clones of
recombinant phages containing an 11.35 kb rDNA
insert in opposite direction

B) λ gtWES Mr974

C) λ gtWES Mr969

the inserted ribosomal fragments within both arms of λ is schematically outlined in Fig. 8. This diagram also summarizes data derived from length measurements of about 30 molecules of the different clones. These length determinations are remarkably consistent with the data obtained by biochemical methods.

Detection of Sequences Complementary to λgtWES Mr100 and 974 in Mouse Genomic DNA

To check whether sequences complementary to the cloned fragments are present only in the ribosomal genes or are distributed elsewhere in the genome we have prepared highly labelled ^{32}P-hybrid phage DNA and used it to monitor the distribution of complementary sequences within mouse DNA. For this,mouse DNA was digested with EcoRI, BamHI,and HindIII,respectively and the sequences complementary to to the cloned fragments were detected by hybridisation.As shown in Fig.9 the sizes of the labelled bands closely corresponds to the distance between the cleavage sites in mouse rDNA published by Cory and Adams(2).The only band which hybridized in the EcoRI digested DNA was the cloned fragment itself. As mentioned before, the inserted fragment in λgtWES Mr974 (11.3 kb) is slightly shorter than the fragment in cellular DNA to which it hybridizes(~14 kb).This means that some deletions must have occured during cloning and propagation of the phage. Uncertain is the origin of the 1.8 kb fragment in the HindIII digest hybridized to the large insert.It could be due to sequence homologies in various regions of the non-transcribed spacer.However, the fact that the main band of this heavily exposed autoradiograph in each case corresponds to the 5' proximal end of the 45S RNA and adjacent spa-
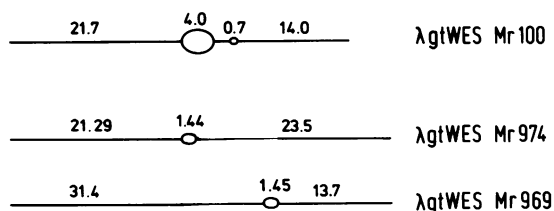

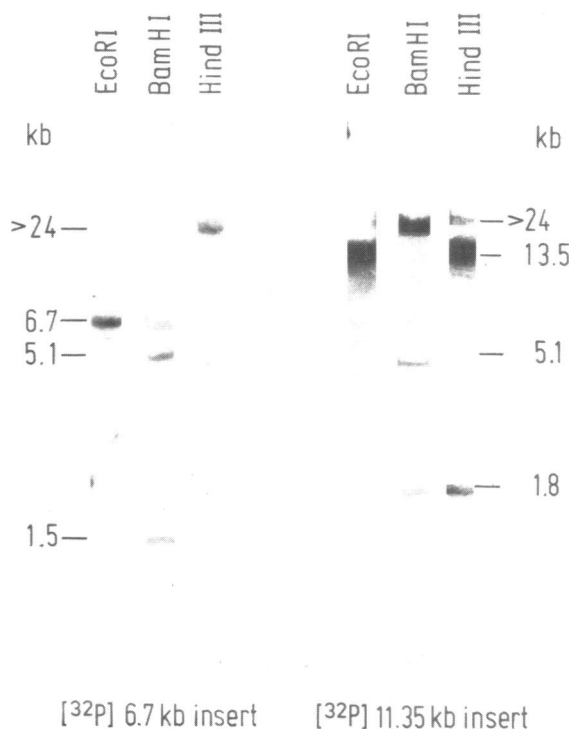Fig. 8 Schematic Representation of R-Loops Formed by the Hybrid Phages with rRNA

Fig. 9 Detection of Fragments Complementary to λgtWES Mr100 and
974 DNA in Total Mouse DNA Digested with EcoRI,BamHI and
HindIII

10 μg of high molecular mouse DNA were digested under standard
conditions with EcoRI,BamHI and HindIII. After ethanol precipi-
tation the samples were fractionated by electrophoresis in a
0.7% agarose gel for 15 h at 15mA. After transfer to Millipore
filters hybridization with nick-translated λgtWES Mr100 and 974
DNA was performed as indicated in Materials and Methods.

cer sequences suggests that the rDNA spacer does not have a sig-
nificant length of sequences in common with other sequences in
the genome.

DISCUSSION

The analysis of the transcription process and the regulation of
the expression of specific genes requires a detailed knowledge
about the organisation of the gene which is to be studied as well
as a potent system which is able to transcribe the gene(s) faith-

fully. Since the transcriptional activity of the ribosomal genes
seems to be regulated predominantly on the level of initiation
(18 -21), cloned DNA fragments containing the initiation site
of transcription might serve as a tool for establishing the re-
gions essential for RNA polymerase binding and probably for con-
trol elements in the DNA. The large fragment generated by EcoRI
cleavage of mouse ribosomal DNA represents a DNA segment with
transcribed and non-transcribed regions, and therefore should
contain those regions essential for the initiation reaction of
transcription.

In this paper the large EcoRI fragment cloned in bacteriophage λ
has been analyzed.The characterisation of the hybrid phage called
λgtWES Mr974 revealed that the inserted DNA is considerably
shorter than the fraction it was cloned from. The clones have
been derived from fractions of a preparative electrophoresis with
a mean size of 14 kb. The inserted fragment, however, is only
11.3 kb in length. This means that some deletions must have oc-
cured during cloning. Preliminary results suggest that such de-
letions occur exclusively in the region of the non-transcribed
spacer. We have observed that different clones differ in the
length of the insert and that during propagation of the phages
frequently smaller inserts arise. Restriction analysis of inserts
of different length revealed an absolute constancy of the codo-
genic region and a considerable variability of the non-transcri-
bed spacer. The length of the shortest insert corresponds close-
ly to the length of the transcribed region (5.7 kb). The unsta-
bility of the non-transcribed spacer suggests that it may repre-
sent a repeating sequence. In this respect it would closely re-
semble the structure of spacers separating the transcribed re-
gions of the 5S RNA genes (22,23) and ribosomal genes of Xenopus
(24,25). Since it has been reported in several cases that high-
ly repeated sequences are unstable due to segregation (26 - 29)
it is reasonable to assume that the instability and heterogene-
ity of the inserted sequence in the hybrid phages containing the
large EcoRI fragment of mouse rRNA is a consequence of excision
of repetitive blocks within the spacer.

At present it is too early to speculate on the nature and role

of the DNA sequence preceeding the 45S RNA transcription unit.
Current work in this laboratory is to localize the 5' end of
the 45S pre-rRNA on the cloned DNA fragment and to characterize
sequences neighbouring the start of the transcriptional unit to
determine the physiological significance of these sequences, in
terms of regulatory and promotor signals and sites important for
initiation of transcription.

REFERENCES

1) Gaubatz, J., Praschad, N., and Cutler, R.G. (1976)
   Biochim. Biophys. Acta 418, 358-375.
2) Cory,S. and Adams,J. (1977) Cell 11, 795-905.
3) Tiemeier, D.C., Tilghman, S.M. and Leder, P. (1977)
   Gene 2, 173-191.
4) Polsky, F., Edgell, M.H., Seidman, J.G., and Leder, P.
   (1978) Anal. Biochem. 87, 397-410.
5) Leder, P., Tiemeier, D., and Enquist, L. (1977)
   Science 196, 1175-1177.
6) Benton, W.D., and Davis, R.W. (1977) Science 196,
   180-182.
7) Kirby, K.S. (1965) Biochem. J. 96, 266-269.
8) Commerford, S.L. (1971) Biochemistry 10, 1993-2000.
9) Gross-Bellard, M., Oudet, P. and Chambon, P. (1973)
   Eur. J. Biochem. 36, 32-38.
10) Maniatis, T., Jeffrey, A., Kleid, D.G. (1975) Proc. Nat.
    Acad. Sci. USA 72, 1184-1188.
11) Philippsen, P., Kramer, R.A. and Davis, R.W. (1978)
    J. Mol. Biol. 123, 371-386.
12) McDonell, M.W., Simon, M.N. and Studier, F.W. (1977)
    J. Mol. Biol. 110, 119-146.
13) Southern, E.M. (1975) J. Mo. Biol. 98, 503-517.
14) Denhardt, D. (1966) Biochem. Biophys. Res. Commun. 23,
    641-696.
15) Wellauer, P.K. and Dawid, J.B. (1977) Cell 10, 193-212.
16) Tiemeier, D.C., Tilghman, S.M., Polsky, F.I., Seidmann, J.G.,
    Leder, A., Edgell, M.H., and Leder, P. (1978) Cell 4,
    237-245.
17) Alwine, J.C., Kemp, D.J., and Stark, G.R. (1977) Proc.
    Nat. Acad. Sci. USA 74, 5350-5354.
18) Lampert, A. and Feigelson, P. (1974) Biochem. Biophys.
    Res. Commun. 58, 1030-1038.

19) Chesterton, C.J., Coupar, B.H.E., Butterworth, P.H.W., Buss, J., and Green, M.H. (1975) Eur. J. Biochem. $\underline{57}$, 79-83.

20) Grummt, I., Smith, V.A., and Grummt, F. (1976) Cell $\underline{7}$, 439-445.

21) Gross, K.J., and Pogo, A.O. (1976) Biochemistry $\underline{15}$, 2082-2086.

22) Brownlee, G.G., Cartwright, E.M. and Brown, D.D. (1974) J. Mol. Biol. $\underline{89}$, 703-718.

23) Fedoroff, N.W. and Brown, D.D. (1978) Cell $\underline{13}$, 701-716.

24) Wellauer, P.K., Dawid, J.B., Brown, D.D., and Reeder, R.H. (1976) J. Mol. Biol. $\underline{105}$, 461-486.

25) Botchan, P., Reeder, R.H., and Dawid, J.B. (1977) Cell $\underline{11}$, 599-607.

26) Hershey, N.D., Conrad, S.E., Sodja, A., Yen, P.H. Cohen, M., Davidson, N., Ilgen, C., and Carbon, J. (1977) Cell $\underline{11}$, 585-598.

27) Brutlag, D., Fry, K., Nelson, T., and Hung, P. (1977) Cell $\underline{10}$, 509-519.

28) Cramer, J.H., Farrelly, F.W., Barnitz, J.T., and Rownd, R.H. (1977) Molec. Gen. Genet. $\underline{151}$, 229-244.

29) Cohen, A., Ram, D., Halvorson, H.O., and Wensick, P.C. (1978) Gene $\underline{3}$, 135-147.