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Replication and Transcription of Eukaryotic DNA in *Escherichia coli*

(restriction/plasmid/transformation/recombination/ribosomal DNA)

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ABSTRACT **Fragments of amplified *Xenopus laevis* DNA, coding for 18S and 28S ribosomal RNA and generated by *Eco*RI restriction endonuclease, have been linked *in vitro* to the bacterial plasmid pSC101; and the recombinant molecular species have been introduced into *E. coli* by transformation. These recombinant plasmids, containing both eukaryotic and prokaryotic DNA, replicate stably in *E. coli*. RNA isolated from *E. coli* minicells harboring the plasmids hybridizes to amplified *X. laevis* rDNA.**

Recombinant DNA molecules constructed *in vitro* from separate plasmids (1, 2) by the joining of DNA fragments having cohesive termini (3, 4) generated by the *Eco*RI restriction endonuclease (5, 6) can form biologically functional replicons when introduced into *Escherichia coli* by transformation (7). The *E. coli* tetracycline resistance plasmid, pSC101 (1, 8) (molecular weight 5.8×10^6), is useful for selection of recombinant plasmids in *E. coli* transformants, since insertion of a DNA segment at its single *Eco*RI cleavage site does not interfere with expression of its tetracycline resistance gene(s) or with the replication functions of the plasmid (1, 2).

This report describes the *in vitro* linkage of pSC101 and eukaryotic DNA cleaved by *Eco*RI endonuclease, and subsequent recovery of recombinant DNA molecules from transformed *E. coli* in the absence of selection for genetic properties expressed by the eukaryotic DNA. The amplified rDNA (coding for 18S and 28S ribosomal RNA) of *Xenopus laevis* was used as a source of eukaryotic DNA, since it has been well characterized and can be isolated in quantity (9, 10). Recombinant plasmids containing both *X. laevis* and pSC101 DNA replicate stably in *E. coli*, where they are capable of synthesizing RNA complementary to *X. laevis* rDNA.

MATERIALS AND METHODS

DNA coding for ribosomal RNA of *X. laevis*, isolated by CsCl-gradient centrifugation, and ^{32}P-labeled 18S and 28S *X. laevis* ribosomal RNA were the generous gifts of Dr. D. D. Brown. Bacterial strains and the tetracycline resistance plasmid pSC101 have been described (1, 2, 8). Covalently-closed circular plasmid DNA was isolated as described (8, 11), or by an adaptation of a NaCl-sodium dodecyl sulfate cleared-lysate procedure (12, 13). Transformation of *E. coli* by plasmid DNA (7), isolation of *E. coli* minicells (14), heteroduplex analysis by electron microscopy (15), DNA·RNA hybridization (16, 17), and analysis of fragments generated by *Eco*RI endonuclease by agarose gel electrophoresis (refs. 1, 6, and 18; Helling, Goodman and Boyer, in preparation) have been described elsewhere. Molecular weights of fragments were calculated from their mobility in gels relative to the mobility of fragments of λ DNA cleaved by *Eco*RI endonuclease. Radioactive labeling of RNA in *E. coli* minicells was according to Roozen *et al.* (19); ^{3}H-Labeled RNA was isolated from minicells by a modification of a procedure described (17).

Purification of *Eco*RI restriction endonuclease (20) and *E. coli* ligase (the generous gift of Drs. P. Modrich and I. R. Lehman) (21) have been described. *E. coli–X. laevis* recombinant plasmids were constructed *in vitro* as follows: the reaction mixture (60 μl) contained 100 mM Tris·HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1.0 μg of pSC101 plasmid DNA, 2.5 μg of *X. laevis* rDNA, and excess *Eco*RI restriction endonuclease (1 μl, 2 units). After a 15-min incubation at 37°, the reaction mixture was placed at 63° for 5 min to inactivate the *Eco*RI endonuclease. A 3-μl sample was examined by electron microscopy to assess digestion. The remainder was refrigerated at 0.5° for 24 hr to allow association of the short cohesive termini; melting temperature (Tm) was 5–6° (3).

The reaction mixture for ligation of phosphodiester bonds was adjusted to a total volume of 100 μl and contained, in addition to the components of the endonuclease reaction, 30 mM Tris·HCl (pH 8.1), 1 mM sodium EDTA, 5 mM $MgCl_2$, 3.2 nM NAD, 10 mM $(NH_4)_2SO_4$, 5 μg of bovine-serum albumin, and 9 units of *E. coli* DNA ligase (21). All components were chilled to 0.5° before their addition to the reaction mixture. Ligase reactions were incubated at 14° for 45 min, and returned to 0.5° for 48 hr. Additional NAD and ligase were added, and the mixture was incubated at 15° for 30 min and then for 15 min at 37°. A 3-μl sample of the mixture was examined by electron microscopy for reassociation of fragments. Ligated DNA was used directly in the plasmid transformation procedure (7).

RESULTS

Cleavage of rDNA of X. laevis. Linear molecules of *X. laevis* rDNA (molecular weight about 50×10^6, as determined by electron microscopy) were treated with excess *Eco*RI endonuclease. After complete digestion, about 44% of the molecules had a molecular weight of 3.1×10^6 (Fig. 1) and a second major class (25%) of fragments had a molecular weight of 4.3

Abbreviations: rRNA, ribosomal RNA; rDNA, amplified DNA containing the genes for 18S and 28S rRNA; *Eco*RI, the RI restriction and modification host specificity of *E. coli* controlled by the fi$^+$ plasmid, pHB1.

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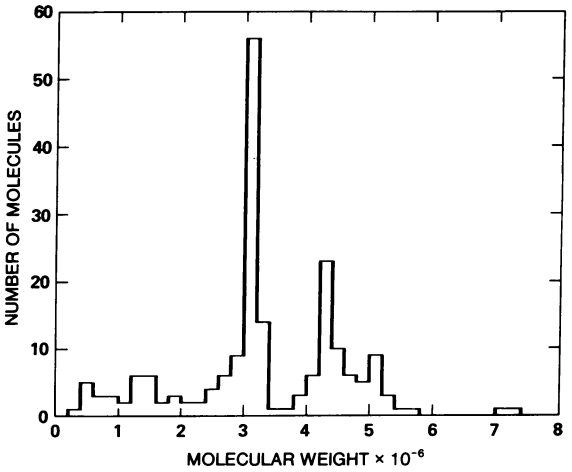

FIG. 1. Histogram of molecular weights of fragments of *X. laevis* rDNA generated by *Eco*RI endonuclease. *X. laevis* rDNA was completely digested by *Eco*RI endonuclease and mounted for electron microscopy. 195 molecular lengths were measured with a Philips EM-300 electron microscope. Molecular weights, which have been multiplied by 10^6, were calculated from lengths of fragments relative to a simian virus 40 (SV40) DNA standard (molecular weight, 3.4×10^6) included on the same grid.

$\times 10^6$ (Fig. 1). In addition, some fragments (9%) having molecular weights of 5.1×10^6 were observed. The occurrence of molecules smaller than 3.1×10^6 may result from length heterogeneity of the untreated rDNA preparation.

Separation of *X. laevis* rDNA cleaved by *Eco*RI endonuclease by agarose gel electrophoresis (Fig. 2) confirmed the electron microscope data and identified other size classes of fragments. Fragments equivalent to the two major DNA lengths seen by electron microscopy were observed in the gels (molecular weight estimates from gel, 3.0×10^6 and 4.2×10^6), and two minor bands having estimated molecular weights of 3.9×10^6 and 4.8×10^6 were seen. Additional bands, which are not readily apparent in Fig. 2, were observed in the original gel. Cleavage of the pSC101 plasmid DNA by the *Eco*RI endonuclease occurred at one site, resulting in formation of a single linear fragment having a molecular weight of about 5.8×10^6 (Fig. 2 and refs. 1 and 2).

Analysis of Recombinant Plasmids. A mixture of pSC101 DNA and *X. laevis* rDNA, both treated with *Eco*RI endonuclease, was ligated after random association of the short cohesive termini had occurred. This DNA was used to transform *E. coli* strain C600 $r_K^- m_K^-$, and tetracycline-resistant transformants ($3.3 \times 10^3/\mu g$ of pSC101 DNA) were selected and numbered consecutively CD1, CD2, etc. Plasmid DNA isolated from three of the transformants yielded three DNA fragments after digestion with the *Eco*RI endonuclease, and ten yielded two fragments (Table 1, Fig. 2). Each plasmid contained a fragment corresponding to linear pSC101 DNA (5.8×10^6, molecular weight). The other fragments had molecular weights (estimated from gels) of 4.2×10^6, 3.9×10^6, or 3.0×10^6, which correspond to the molecular weight estimates for certain of the *X. laevis* rDNA fragments generated by the endonuclease. An example of each type of recombinant plasmid was selected for further study.

Buoyant densities of *X. laevis* rDNA and of *Eco*RI digests of these four representative recombinant plasmids were compared (Fig. 3). Each of the plasmid DNA species contains a fragment generated by *Eco*RI endonuclease having a buoyant density about equal to that of amplified *X. laevis* rDNA ($\rho = 1.729$ g/cm^3) (29), in addition to a fragment having the buoyant density of pSC101 DNA ($\rho = 1.710$ g/cm^3). We infer from the similar buoyant densities of the three *X. laevis* rDNA

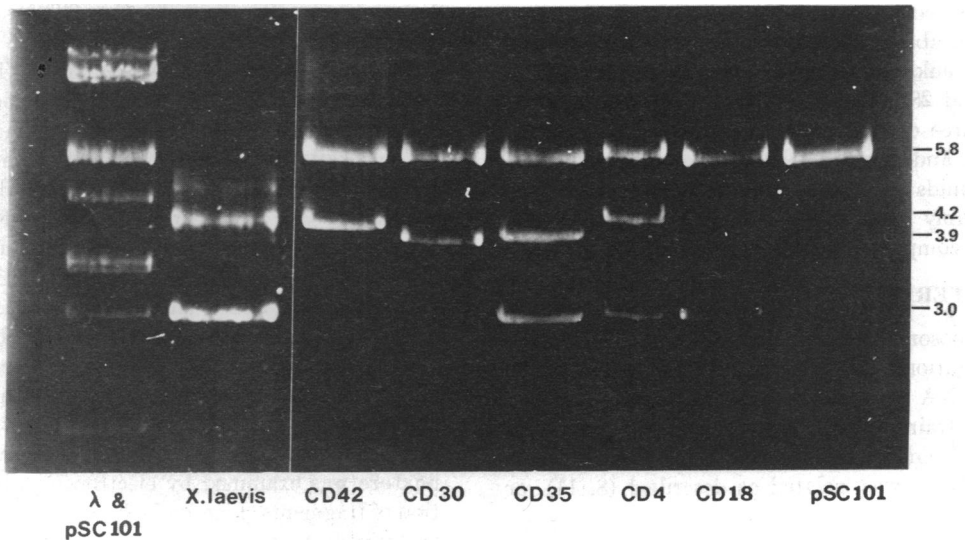

FIG. 2. Agarose gel electrophoresis of fragments generated by *Eco*RI endonuclease. DNA was isolated and digested to completion with excess *Eco*RI endonuclease. Electrophoresis was in a 0.7% agarose slab gel (17 cm × 13 cm × 3 mm) at 3 V/cm in 0.09 M Tris base–2.8 mM EDTA–0.09 M boric acid (pH 8.3) buffer at 25° for 15 hr. After completion of electrophoresis, the DNA was stained with 4 μg/ml of ethidium bromide and photographed under a long-wave UV lamp (refs. 1, 6, and 18; Helling, Goodman and Boyer, in preparation). Electrophoresis was from top to bottom in the figure (i.e., the anode is at the *bottom*) and the source of DNA is indicated. The figure shown is a composite of two separate gels. An *Eco*RI endonuclease digest of a mixture of bacteriophage λ DNA and pSC101 DNA was used for molecular weight standardization, and is shown in the *left-hand column*. The bands seen in this column had estimated molecular weights of (counting from the *bottom*): 2.09, 3.03, 3.56, 3.7, 4.7, 5.8 (pSC101), 13.7, and 15.7, all $\times 10^{-6}$ (Helling, Goodman and Boyer, in preparation). The 15.7×10^6 fragment results from joining of the terminal cohesive ends of λDNA, which are located on the 2.09 and 13.7 $\times 10^6$ fragments. The calculated molecular weights of the major fragments present in *X. laevis* rDNA treated with *Eco*RI endonuclease and in the plasmids shown are indicated in the figure. All values have been multiplied by 10^{-6}.

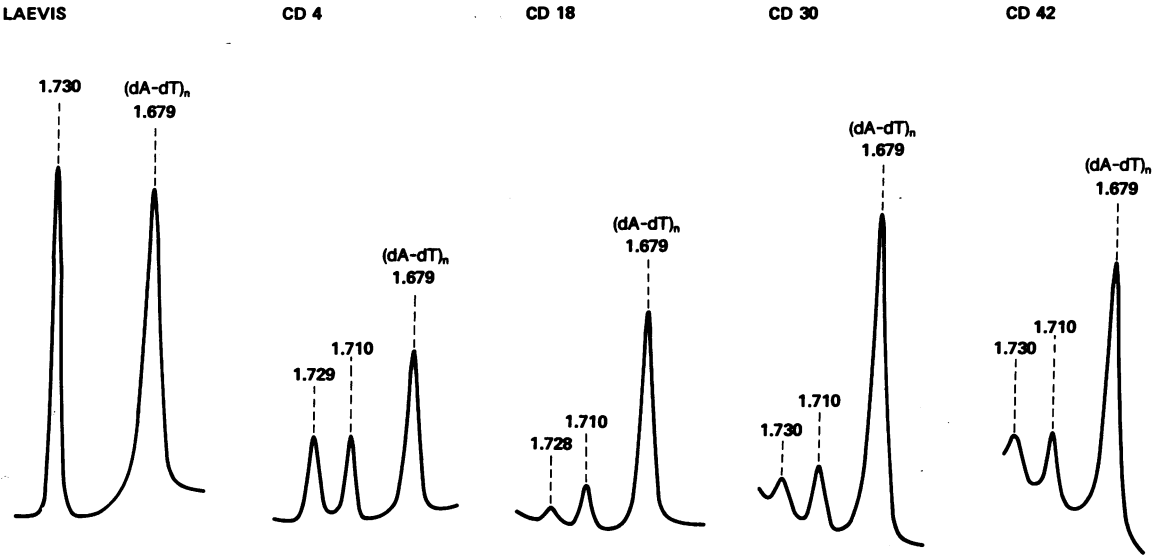

FIG. 3. Analytical ultracentrifugation of DNA, cleaved by *Eco*RI endonuclease, isolated from *E. coli* transformants carrying various recombinant plasmids and *X. laevis* rDNA. Covalently-closed circular plasmid DNA was cleaved as indicated in *Methods* and Fig. 2. Centrifugation in CsCl (ρ = 1.710 g/cm^3) was for 28–36 hr at 44,000 rpm in the presence of (dA-dT)$_n$(ρ = 1.679 g/cm^3) density marker. Densitometer tracings of photographs taken during centrifugation are shown.

fragments present in the separate plasmids, that *Eco*RI endonuclease cleavage of *X. laevis* rDNA produces fragments that are similar to each other in (G+C) composition. However, we did not have a sufficient amount of rDNA to carry out this analysis directly.

Electron microscope analysis of a heteroduplex formed between *X. laevis* rDNA and the plasmid CD42 (Fig. 4) shows that the plasmid contains DNA nucleotide sequences present in *X. laevis* rDNA. Moreover, in this and other heteroduplexes, two separate plasmid DNA molecules were seen to form duplex regions with a single strand of *X. laevis* rDNA, consistent with the observation (22, 23) that the rDNA sequences of *X. laevis* are tandemly repeated.

TABLE 1. *X. laevis–E. coli recombinant plasmids*

Plasmid DNA	Molecular weight of *Eco*RI plasmid fragments estimated by gel electrophoresis ($\times 10^{-6}$)	Molecular weight from contour length ($\times 10^{-6}$)	Buoyant density in CsCl (g/cm^3)
Tetracycline-resistant clone			
CD4	5.8, 4.2, 3.0	13.6	1.721
CD7	5.8, 4.2	—	—
CD12, CD20, CD45, CD47, CD51	5.8, 3.0	—	—
CD14	5.8, 4.2, 3.0	—	—
CD18	5.8, 3.0	9.2	1.720
CD30	5.8, 3.9	10.0	1.719
CD35	5.8, 3.9, 3.0	—	—
CD42	5.8, 4.2	10.6	1.720
pSC101	5.8	6.0	1.710

The procedures used for isolation of DNA, agarose gel electrophoresis, CsCl gradient centrifugation, and calculation of a molecular weight and buoyant density are indicated in *Methods*. The reproducibility of molecular weight estimates from the gel mobility of DNA fragments is ±5%. The standard deviation of contour lengths used for molecular weight estimates was 2–4%.

^{32}P-Labeled 18S and 28S *X. laevis* rRNA were hybridized to DNA obtained from the plasmids CD4, CD18, CD30, and CD42 (Fig. 5). CD4 DNA, which contains both the 3.1 $\times$ 10^6 and 4.3 $\times$ 10^6 fragments of *X. laevis* rDNA, anneals almost equally with both the 18S and 28S rRNA species. CD18 plasmid DNA hybridizes principally with 28S *X. laevis* rRNA, while the DNA of plasmids CD30 and CD42 anneals primarily with 18S ribosomal RNA. These data suggest that the latter two plasmids contain DNA fragments having a similar nucleotide sequence and suggest that the *X. laevis* rDNA fragment of CD30 may lack a short sequence contained in the 4.3 $\times$ 10^6 fragment carried by CD42. Contamination of the 18S rRNA preparation with 28S rRNA may account for the small amount of hybridization of 18S [^{32}P]RNA observed with the DNA of the CD18 plasmid, which carries only the 3.0 $\times$ 10^6 *Eco*RI fragment of *X. laevis* rDNA.

Transcription of X. laevis DNA in E. coli Minicells. It has been reported that various plasmids can segregate into *E. coli* minicells (14, 19, 24, 25) at the time when these spheres are budded off from the parent *E. coli* (26). The lack of chromosomal DNA in minicells has made them particularly suitable for studies of plasmid DNA, RNA, and protein synthesis (19, 27, 28) in the absence of a background of chromosomal macromolecular synthesis. The minicell-producing *E. coli* strain P678-54 was transformed to tetracycline resistance with plasmid DNA isolated from C600 $r_K^-m_K^-$ containing CD4, CD18, or CD42. Transformation as well as transfer of recombinant plasmids to this and to other $r_K^+m_K^+$ strains occurred at a frequency reduced by 50- to 1000-fold, compared with the $r_K^-m_K^-$ strain. Minicells containing the plasmids were isolated and incubated with [^{3}H]uridine; RNA purified from such minicells was hybridized with *X. laevis* rDNA immobilized on nitrocellulose membranes, in order to determine whether the *X. laevis* rDNA linked to the pSC101 replicon

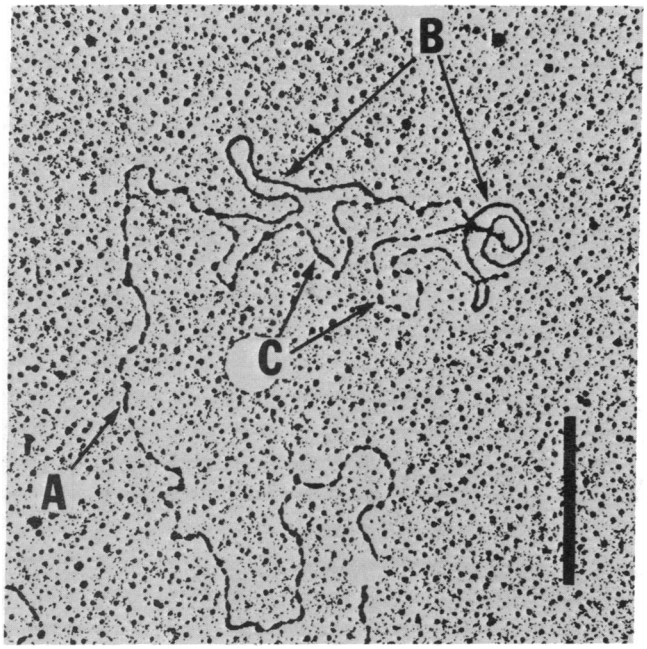

FIG. 4. Electron photomicrograph of heteroduplex of *X. laevis* rDNA and two separate CD42 plasmid DNA molecules. (*A*) Single strand of *X. laevis* rDNA. (*B*) Double-stranded regions of homology between plasmid CD42 and *X. laevis rDNA*. (*C*) Single-stranded regions corresponding in length to the DNA segment of CD42 derived from pSC101. CD42 DNA was nicked by x-irradiation (0.8 break/strand) to permit strand separation. The molecular weight of the double-stranded heteroduplex region is 4.5×10^6, relative to CD42 DNA standard on the same grid (12 measurements, standard deviation 0.25×10^6). The contour length of the DNA segment separating the two duplex regions is consistent with a single-strand molecular weight of 1.5×10^6 for this segment (2 measurements). The *bar* indicates 1 μm. About one-third of *X. laevis* rDNA single strands hybridized with one or more molecules of CD42 DNA.

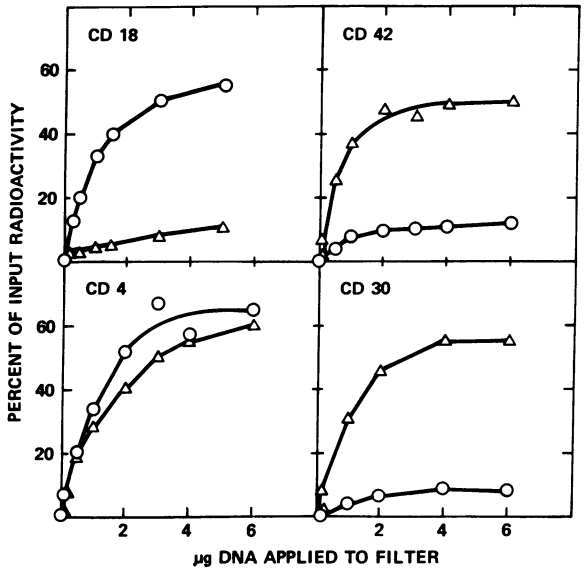

FIG. 5. Hybridization of *X. laevis* ^{32}P-labeled ribosomal RNA (specific activity 9×10^6 cpm/μg) with DNA of recombinant *X. laevis–E. coli* plasmids. (△) 18S ribosomal RNA; (○) 28S ribosomal RNA. Hybridization on nitrocellulose membranes was carried out at DNA excess. Filters containing 2 μg of pSC101 DNA bound less than 0.1% of input RNA under the experimental conditions used. 900 cpm (0.01 μg) input [^{32}P]RNA was used for each filter.

is transcribed in *E. coli*. These results (Table 2) show that RNA species capable of annealing with purified *X. laevis* rDNA are synthesized in *E. coli* minicells carrying the recombinant plasmids CD4, CD18, and CD42, but not by minicells carrying the pSC101 plasmid alone.

DISCUSSION

The presence of *X. laevis* rDNA replicating as a part of a bacterial plasmid in *E. coli* has been demonstrated by five separate criteria: (*1*) Treatment of recombinant plasmid DNA molecules with the *Eco*RI restriction endonuclease generates fragments that have molecular weights indistinguishable from those of the cleaved *X. laevis* ribosomal DNA and linear pSC101 DNA. (*2*) Uncleaved recombinant plasmids have buoyant densities in CsCl intermediate to the buoyant densities of pSC101 and *X. laevis* rDNA. The plasmid fragments generated by the *Eco*RI restriction enzyme are similar in buoyant density to *X. laevis* rDNA, and reflect the (G+C) content of amplified ribosomal RNA genes from that organism (9, 10). (*3*) Electron microscope heteroduplex analysis of recombinant plasmid DNA molecules isolated from *E. coli* indicates sequence homology with *X. laevis* rDNA. In some instances more than one plasmid molecule was observed to anneal with a single strand of rDNA, which is known to have tandemly repeated sequences. (*4*) 18S and 28S ribosomal [^{32}P]RNA obtained from *X. laevis* anneals with recombinant plasmid DNA molecules isolated from *E. coli*. (*5*) RNA synthesized by *E. coli* minicells carrying recombinant plasmids hybridizes to rDNA from *X. laevis*, providing a functional demonstration of the presence of *X. laevis* rDNA in *E. coli*.

It has been reported (1) that fragments, generated by *Eco*RI endonuclease, of the large antibiotic resistance plasmid R6-5 can randomly associate to form new plasmid DNA species which in turn can be linked to pSC101 *in vitro* and introduced

TABLE 2. [^{3}H]*RNA synthesized by E. coli minicells*

Plasmid carried by minicells	Input cpm	[^{3}H]RNA counts hybridized to *X. laevis* rDNA 0.2 μg	*X. laevis* rDNA 0.4 μg	pSC101 DNA 1.8 μg
CD42	4810	905 (19%)	1436 (30%)	961 (20%)
CD18	3780	389 (10%)	—	1277 (34%)
CD4	5220	789 (15%)	—	1015 (19%)
pSC101	4170	0 (0%)	—	1500 (36%)

Minicells containing plasmids were isolated as described by Cohen *et al.* (14) and were incubated with [^{3}H]uridine (50 μCi/ml, 30 Ci/mol) as described by Roozen *et al.* (19) for 10 min at 37°. Minicells collected by centrifugation were resuspended in Tris·HCl (20 mM, pH 7.5)–5 mM $MgCl_2$–1 mM EDTA, pH 8.0, and rapidly frozen and thawed three times. RNA was extracted as described (17). Hybridization assays were carried out on nitrocellulose membranes as described (16, 17) at saturating levels of pSC101 DNA. Hybridizations involving *X. laevis* DNA were not performed at DNA excess. Counts bound to blank filters (5–10 cpm) were subtracted from experimentally determined values. There is no cross-hybridization between bacterial ribosomal RNA and *X. laevis* rDNA (28). ^{3}H counts eluted from filters containing *X. laevis* DNA were rendered acid-soluble by ribonuclease A (20 μg/ml, 0.30 M NaCl–0.030 M Na citrate, 1 hr, 37°) but were resistant to pancreatic deoxyribonuclease [20 μg/ml, 10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 1 hr, 37°].

into *E. coli* by transformation. These earlier investigations used markers carried by *Eco*RI endonuclease-generated fragments incorporated into the recombinant plasmid to select for transformants. In the present experiments, tetracycline-resistant transformants consisting of pSC101 linked to *X. laevis* were recovered by random association (ratio: 4 *X. laevis* rDNA termini per pSC101 terminus) of DNA fragments in the absence of selection for any *X. laevis* genetic marker. Since the majority (75%) of the tetracycline-resistant clones contain only the pSC101 DNA molecule, the conditions used may favor cyclization of this molecule; tandem pSC101 plasmid DNA molecules were not observed in this recombination-competent host.

Although transcription of *X. laevis* rDNA was detected in *E. coli* C600 transformants (unpublished data), the amount of *X. laevis*-hybridizable material isolated from such cells represented too small a fraction of the total ^{3}H-labeled RNA to be studied easily. *E. coli* minicells thus provide a powerful tool for the investigation of macromolecular synthesis coded by *X. laevis* rDNA and by other eukaryotic genes joined to bacterial plasmids, since minicells are devoid of bacterial chromosomal DNA and plasmid species segregating into minicells are capable of plasmid-specific synthesis of functional gene products (27, 28). Moreover, study of control of eukaryotic gene transcription may be possible in *E. coli* minicells, since mechanisms regulating the repression and derepression of anthranilate synthetase (28) and tetracycline resistance (27) have been shown to be operative in these cells.

Since the *X. laevis* rDNA inserted into the pSC101 plasmid does not code for protein, we do not yet know whether synthesis of proteins can be carried out by eukaryotic DNA in *E. coli*. In addition, it is not clear whether the RNA synthesized on *X. laevis* rDNA template in *E. coli* minicells is an accurate transcript of the eukaryotic genes. However, these experiments demonstrate that transcription of cloned *X. laevis* rDNA does occur in a prokaryotic organism. In addition, the location of an *Eco*RI cleavage site near the boundary between the 18S and 28S rRNA genes (as shown by the data in Fig. 5) suggests that investigation of the transcription of specific *X. laevis* rRNA cistrons in *E. coli* may be practical.

X. laevis rDNA replicates stably in *E. coli* for at least 100 generations (unpublished data) as part of the pSC101 plasmid replicon, and can be recovered from transformed *E. coli* in amounts required for *in vitro* analysis by procedures commonly used for the isolation of bacterial plasmids. Cloned *X. laevis* DNA can then be introduced into other *E. coli* strains by transformation. The ability to clone specific fragments of DNA from a complex genome provides a potentially valuable tool for the study of organization and function of eukaryotic genomes. Our results using cloned *X. laevis* rDNA suggest that the amplified ribosomal RNA genes of this organism may not be contained in homogeneous units of repeated sequences, as previously supposed (9, 10), but that some degree of heterogeneity with respect to the size of the repeat unit may occur in the rDNA sequence. The gel electrophoresis and DNA·RNA hybridization data reported here indicate that at least two *Eco*RI sites are present per *X. laevis* repeat unit and suggest that the 3.0×10^6-dalton fragment generated by *Eco*RI endonuclease may be paired with either the 3.9×10^6 or the 4.2×10^6-dalton fragment in two kinds of commonly occurring repeat units existing in the preparation of *X. laevis* rDNA we have studied. The molecular weight estimated for the intact repeat unit (7.2 to 7.5×10^6) is in general agreement with data obtained by Hourcade *et al.* (23) and by A. Forsheit, N. Davidson and D. D. Brown (personal communication).

The procedure reported here offers a general approach utilizing bacterial plasmids for the cloning of DNA molecules from various sources, provided that both molecular species have cohesive termini made by a restriction endonuclease, and that insertion of a DNA segment at the cleavage site of the plasmid does not interfere with expression of genes essential for its replication and selection.

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