ABSTRACT DNA containing the multiple genes for 5S RNA has been isolated from the genome of *Xenopus laevis*. Whereas 5S RNA is about 57% G + C, the 5S DNA has a base composition of about 33-35% GC and consists of two alternating regions that differ in base composition by at least 20% GC. A denaturation map of 5S DNA analyzed by electron microscopy demonstrates that the repeating pattern is regular and each repeating unit has a mass of about 500,000 daltons. If one gene for 5S RNA (84,000 daltons native) were present in each repeat, it should comprise about 16.8% of 5S DNA. This arrangement is confirmed, since 6.8% of pure 5S DNA (13.6% of its base pairs) hybridized with 5S RNA. The remaining 83% of each repeating unit is considered to be "spacer" DNA. The 5S RNA hybridizes with about 0.05% of the bulk DNA of *X. laevis*, so that 5S DNA comprises about 0.7% of the total nuclear DNA. This is equivalent to about 24,000 repeating units for each haploid complement of DNA. These repeats are highly clustered; as many as 86 have been visualized along a single DNA molecule.

The small RNA component of ribosomes, termed 5S RNA, comprises about 1-2% of total cellular RNA in prokaryotes and eukaryotes. In the South African toad, *Xenopus laevis*, 5S RNA genes are known to be in greater abundance than the genes for 18S and 28S ribosomal RNA and are not intermingled with them (1). This report documents the purification of the DNA containing the genes for 5S RNA from the genome of *X. laevis* and describes some characteristics of 5S DNA.

MATERIALS AND METHODS

Crude CsSO₄ was purchased from Gallard Schlessinger (more than 99% pure, catalogue no. 27573) and was found to be as good for the experiments described here as the much more expensive optical-grade product. CsCl was purchased from Harshaw, and actinomycin D was a gift of Merck. The antibiotic was dissolved in a stock aqueous solution (1 mg/ml) and stored at 4°C in the dark.

Purification of DNA. DNA was purified from isolated nuclei of *X. laevis* erythrocytes (1). To maintain the DNA at the highest possible molecular weight, it was never precipitated during purification. The purified nuclei, suspended in SSC (0.15 M NaCl-0.015 M sodium citrate), were lysed with 0.5% sodium dodecyl sulfate and digested with 1 mg/ml of Pronase overnight at 37°C. The DNA was extracted gently with phenol three or four times. The aqueous phase was then dialyzed against daily changes of 0.01 M sodium borate (pH 9.2) in preparation for Ag⁺-CsSO₄ centrifugation (2, 3). Dialysis was stopped when phenol was no longer detectable by its absorbance at 270 nm. This DNA was concentrated to about 150 μg/ml by evaporation in dialysis bags aided by a stream of cold air (pervaporation). The preparation of [³²P]DNA from cultured cells grown in ³²PO₄, the enzymatic hydrolysis of this DNA to 5'-deoxyribonucleotides, and the separation and quantitation of the resultant deoxyribonucleotides by thin layer chromatography has been described (4).

RNA Purification and Hybridization. Radioactive 5S RNA (5) and 18S and 28S ribosomal RNA (rRNA) (1) were purified from tissue culture cells of *X. laevis* that had been labeled for several days with [³H]uridine or [³²P]PO₄. The specific activities of [³H]- and [³²P]RNAs were about 3 × 10⁶ and 5 × 10⁸ cpm/μg, respectively. The 5S RNA was contaminated slightly with rRNA. However, rRNA hybridization can be distinguished easily from that of 5S RNA (ref. 1 and see Fig. 1a).

For saturation-hybridization experiments, about 1.5 mg of 5S RNA isolated from ovaries of *X. laevis* was methylated in vitro with 5 mCi of [³H]dimethyl sulfate (6). The 5S [³H]RNA was purified by four ethanol preparations that removed all acid-soluble radioactivity; the specific activity of the RNA was 10,000 cpm/μg.

Radioactive RNA was hybridized with DNA immobilized on Millipore HA filters. Aliquots of DNA complexed with silver were mixed with 10 volumes of 20 × SSC to complex the silver before the DNA was denatured and trapped on filters. The hydridization medium contained 50% formamide, 4 × SSC, and 0.05 M Tris (pH 7.8); incubation took place at 40°C overnight (7). The filters were washed, treated with RNase, and counted in a scintillation counter.

Denaturation Map of 5S DNA. The 5S DNA was partially denatured with alkali for electron microscopy (8). The exact procedure that we used for 5S DNA has been described in detail for ribosomal DNA (9).

RESULTS

Purification of 5S DNA. Two characteristics of 5S DNA from *X. laevis* made its isolation possible. First, hybridization experiments have shown that thousands of genes for 5S RNA are present in a haploid complement of *X. laevis* DNA, so that 5S DNA is abundant in the genome (ref. 1, and see Table 2). Second, 5S DNA has a lower buoyant density in CsCl than does the bulk of *X. laevis* DNA (1) however, this difference (about 7 mg/cm³) is not enough to allow its separa-
The density marker was slowly centrifuged in CsCl with Ag+ as an antibiotic. The density marker was also centrifuged in CsSO4 with Ag+ to identify the lightest DNA. Ribo- sonal DNA complexes with the most actinomycin, and hence bands as the lightest DNA (not shown here). The DNA was adjusted to a refractive index of 1.3930 with solid CsCl, and stock actinomycin D (1 mg/ml) was added to a final ratio of 2:1 (w/w DNA:actinomycin D). Centrifugation was 35,000 rpm in a no. 65 fixed-angle rotor for 40 hr at room temperature. Actinomycin was removed from purified SS DNA by extensive dialysis against 20x SSC, followed by dialysis against 1x SSC.

The extent of purification after each step was monitored by CsCl analytical ultracentrifugation (Fig. 2). The final yield of SS DNA was usually about 0.04-0.07% of the starting material. This is a yield of about 6-10%. Yields could be doubled by careful collection of the SS DNA peak from the first Ag+-CsSO4 gradient step, then directly performing the actinomycin step.

Table 1. Base composition of bulk [3H]DNA and SS DNA

<table>
<thead>
<tr>
<th></th>
<th>Bulk DNA</th>
<th>SS DNA*</th>
<th>SS RNA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>27.8</td>
<td>35.1</td>
<td>20.6</td>
</tr>
<tr>
<td>T(UMP)</td>
<td>32.9</td>
<td>30.3</td>
<td>21.8</td>
</tr>
<tr>
<td>GMP</td>
<td>19.8</td>
<td>16.9</td>
<td>30.4</td>
</tr>
<tr>
<td>CMP</td>
<td>17.6</td>
<td>15.7</td>
<td>27.2</td>
</tr>
<tr>
<td>5-MethylCMP</td>
<td>1.9</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td>% GC content</td>
<td>39.3</td>
<td>34.6</td>
<td>57.6</td>
</tr>
</tbody>
</table>

* The DNAs were hydrolyzed enzymatically to 5'-deoxy-nucleotides, which were separated by thin-layer chromatography. A single radioautogram was traced to determine each base composition (4).

† Refs. 12 and 20.
TABLE 2. Saturation of purified 5S DNA and bulk X. laevis DNA with 5S [3H]RNA

<table>
<thead>
<tr>
<th></th>
<th>μg of RNA/μg of DNA × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified 5S DNA</td>
<td>6.8 ± 1.2*</td>
</tr>
<tr>
<td>Bulk X. laevis DNA</td>
<td>0.05†</td>
</tr>
</tbody>
</table>

* Concentrations of RNA ranging from 7 to 72 μg/ml of RNA gave similar values; therefore, all values were averaged and the standard deviation is given. The amount of DNA (about 2 μg) adsorbed to each filter (1.3-cm diameter) was determined by the absorbance at 260 nm released after hydrolysis at 80°C for 30 min in 0.5 ml of 1 N HCl (1).
† Saturation measurements were made on alkaline CsCl gradients of about 100 μg of bulk X. laevis DNA. Only counts bound in the low buoyant-density part of the gradient were scored. This additional specificity makes it possible to eliminate hybridization of RNA contaminants, particularly rRNA. (See ref. 1 for a discussion of this method.) Concentrations of RNA ranged from 5 to 25 μg/ml; RNA hybridized with 0.04–0.055% of the genome.

Base Composition of 5S DNA. The buoyant density of 5S DNA (1.692 g/cm³) corresponds to about 33% GC (11), a value considerably different from the base composition of 5S RNA (57% GC (12, 20)). A single base-composition analysis has been performed on purified 5S [32P]DNA isolated from tissue culture cells of X. laevis (Table 1). Although additional measurements will be required to give an exact base composition, two features of the DNA are obvious. First, as predicted by its buoyant density it has a lower GC content than bulk DNA. Second, the content of 5-methyldeoxycytidylic acid is about the same in 5S DNA as in bulk DNA and, therefore, cannot account for the low buoyant density of 5S DNA (4, 13).

The strands of 5S DNA differ in density by 23 mg/cm³ in alkaline CsCl (Fig. 3). The light strand is complementary to 5S RNA. Analysis of complementary [32P]RNA transcribed from 5S DNA with Escherichia coli RNA polymerase that was hybridized to the separated strands of 5S DNA has demonstrated that the heavy strand has a high content of T and G relative to the light strand (unpublished observations).

Saturation Hybridization of 5S DNA and Bulk DNA of X. laevis with 5S RNA. The fraction of 5S DNA homologous to 5S RNA was determined by saturation hybridization of purified 5S DNA and bulk X. laevis DNA (Table 2). About 6.8% of purified 5S DNA (13.6% of the base pairs) and about 0.05% of unfractonated bulk DNA hybridized with 5S[3H]-RNA. From these values, we estimate that 5S DNA comprises about 0.7% of bulk X. laevis DNA, which includes about 24,000 genes for 5S RNA for each haploid complement of DNA.

Melting Curve and the Denaturation Map of 5S DNA. The low buoyant density of 5S DNA was at least partly accounted for when its thermal denaturation profile was analyzed (Fig. 4). The melting curve is biphasic, with a long plateau that divides it into two phases. The Tm of the first transition (which comprises about 55% of the total hyperchromicity) is 67°C in 0.1 × SSC, while the Tm of the second transition is 76°C. The difference in these Tm values corresponds to a GC difference of greater than 20%. Because of its high GC content, we presume that the DNA sequence coding for 5S RNA is the last region of 5S DNA to denature; the 5S sequence should comprise about one-third of the second hyperchromicity rise.

The regular arrangement along 5S DNA of the sequences with different GC content is demonstrated by a "denaturation map", as visualized in the electron microscope (Fig. 5). When 5S DNA is about half-denatured, it exhibits a pattern of regularly alternating native and denatured regions along its entire length. As many as 86 of these repeats have been found in a single molecule; the number appears to be limited only by the lengths of DNA we can isolate. The average repeat-length (the distance from the beginning of one denatured region to the next) was about 0.29 μm. From the contour

TABLE 3. Repeat length of 5S DNA

<table>
<thead>
<tr>
<th>Repeat length</th>
<th>Daltons × 10⁵ Range × 10⁶</th>
<th>No. of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured region</td>
<td>0.50 ± 0.07</td>
<td>0.24–0.83</td>
</tr>
<tr>
<td>alone</td>
<td>0.30 ± 0.06</td>
<td>0.12–0.43</td>
</tr>
</tbody>
</table>

The mass was computed by converting contour length to daltons. Tₘ DNA molecules on the same grids were measured and assumed to be 25 × 10⁶ daltons (19). The linear density of DNA was 1.78 × 10⁶ daltons/μm. The limit of measurement of DNA lengths is about 0.02 μm (35,000 daltons), and accounts in part for the high standard deviation for these short lengths.

Fig. 3. Alkaline CsCl equilibrium centrifugation of purified 5S DNA. About 1 μg of purified 5S DNA was centrifuged for 18 hr at room temperature at 44,000 rpm. The DNA was dissolved in 0.1 N KOH-5 mM K₂HPO₄-5 mM EDTA. The densities were calculated with E. coli as a standard (1.772 g/cm³).

Fig. 4. Thermal-denaturation profile of purified 5S DNA. The 5S DNA in 0.1 × SSC, at a concentration of 20 μg/ml, was denatured in a Gilford spectrophotometer with a temperature recorder. The arrow denotes the Tm of B. subtilis DNA (43% GC) in the same solvent.
length of intact phage T7 DNA on the same grids, this length was calculated to correspond to 500,000 daltons (Table 3).

**DISCUSSION**

It has been possible to isolate 5S DNA because of its abundance in the genome of *X. laevis* and its unusual behavior upon buoyant density centrifugation (1). This behavior is not only due to its low GC content. Indeed, 5S DNA has in common with the high-GC content DNA that codes for ribosomal RNA the fact that both bind less Ag⁺ than the bulk of *X. laevis* DNA (Fig. 1a). This is also true of at least two other "satellite" DNAs of *X. laevis*, and at least part of the DNA homologous to 4S RNA (unpublished observations). We do not know what properties these DNAs share that would account for this interaction beyond the fact that they are all presumably repetitious DNAs. Similar purification techniques work with various satellites of eukaryotic DNA (3). The efficacy of the actinomycin purification can be explained by the low affinity of the drug for high-AT content DNA (10). The 5S DNA behaves as a homogeneous DNA component when bulk *X. laevis* DNA is complexed with silver or mercury and banded in Cs₂SO₄, or when it is banded in neutral or alkaline CsCl, or when it is complexed with actinomycin D in neutral CsCl. For this reason, the low yield of 5S DNA (about 7%) was not due to heterogeneity of 5S DNA sequences but rather to losses incurred by the many steps required for 5S DNA purification. For example, major losses are taken in the first Cs₂SO₄ gradient due to the high concentration of DNA, which causes trapping of some 5S DNA within the main band of DNA. This trapping is an
artifact of DNA concentration, since lower starting concentrations (20 μg/ml or less) obviate the problem.

The single direct measurement of base composition (Table 1) and buoyant density (Fig. 2) of 5S DNA estimate its base composition to be 33–35% GC. This overall value is the average of two regions with vastly different base compositions (Fig. 4). One repeating unit of 5S DNA containing one low-GC and one high-GC region has an average mass of 500,000 daltons (Table 3). The 5S RNA is encoded in about 13.6% of the base pairs of 5S DNA (Table 2). The calculated molecular weight of the 120 base-pairs of DNA which code for one 5S RNA molecule (12) is about 54,000, or about 16.8% of one repeat-length of 500,000. The agreement of these numbers (13.8 and 16.8%) leads us to conclude that each repeating unit contains a single sequence coding for 5S RNA. This sequence is probably the stretch within 5S DNA of highest GC content.

The DNA containing 5S genes is the second DNA of known function to be isolated from eukaryotic DNA, the first being the ribosomal DNA that includes the genes for 18S and 28S rRNA of X. laevis (14). The 5S DNA resembles rDNA in several ways. It is highly repetitive, and the DNA sequences that are known to function in the living cell are separated by spacer regions. Whereas slightly more than half of each repeating unit of the rDNA of X. laevis is transcribed in vivo (4, 9), only about one-sixth of each repeat within 5S DNA is transcribed as 5S RNA. We do not know whether additional sequences of 5S DNA are transcribed in vivo, perhaps as part of a high molecular weight precursor to 5S RNA. Two kinds of evidence from studies of mammalian 5S RNA are relevant to this possibility. First, a fraction of newly synthesized 5S RNA isolated from HeLa cells has triphosphate at the 5’-end of the molecule (15). These molecules are otherwise identical to the bulk of 5S RNA. This finding suggests that no additional nucleotide was transcribed at the 5’-end of the molecule in vivo. Second, with short pulse-labels of nucleotides, when precursor forms of rRNA and tRNA predominate, mature 5S RNA has been synthesized (16). These two facts favor the idea that there may not be a precursor to 5S RNA in animal cells.

The repeated sequences of 5S DNA are at least partly clustered. However, we do not know whether all 5S DNA is clustered on a single chromosome of X. laevis, as is rDNA (14). 5S DNA is not intermingled with rDNA in X. laevis (1), although the two sequences of DNA could be located on the same chromosome. In HeLa cells, chromosome fractionation studies have indicated that the 5S genes are located on more than one chromosome (17). In contrast, the 5S genes are clustered in the Drosophila genome (18).

Another feature of 5S DNA that is under investigation is the regularity and homogeneity of the multiple 5S DNA repeats. The repeat lengths are very uniform (Table 3). Out of several-hundred denatured regions that were measured (Table 3), the highest value was less than twice the mean value. We conclude that two such low-GC regions are rarely, if ever, adjacent. Despite the fact that 5S RNA is encoded by about 24,000 genes for each haploid complement of DNA, these multiple gene sequences must be very similar, if not identical. This conclusion is derived from preliminary sequence studies of 5S RNA (20). The oligonucleotide patterns obtained from RNase digests are compatible with a unique sequence for X. laevis 5S RNA. In contrast, our preliminary experiments suggest that the multiple spacer sequences of X. laevis 5S DNA, while homogeneous in length, contain considerable heterogeneity in their nucleotide sequences (unpublished observations).

We are grateful to Drs. I. Dawid, L. P. Gage, R. Reeder, and R. Roeder for their helpful criticisms of the manuscript. We are indebted to Mr. R. Stern who first suggested that we use actinomycin for 5S DNA purification.