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# CLEAVAGE SITES OF RESTRICTION ENDONUCLEASES *Eco*RI, *Bam*HI AND *Hin*dIII ON CHICKEN EMBRYO rDNA

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## 1. Introduction

Recent studies on ribosomal genes in *Xenopus laevis* have shown, that the genes for 18 S and 28 S rRNA are arranged in tandem repeats with length heterogeneity of the untranscribed spacer [1–3]. In *Drosophila melanogaster* two types of repeats have been found, one of them bearing an insertion of varying length within the 28 S rRNA gene [4–8]. Experimental data from the cleavage sites of different restriction enzymes on ribosomal DNA in higher eukaryotes, like mouse [9,10] or man [9], have indicated, that the relative length heterogeneity of the repeat unit for these organisms is not so extensive. On the other hand, a minor heterogeneity of restriction fragments was detected, when the DNA was isolated from different individuals [9]. Ribosomal genes of chicken are present in ~200–240 copies/haploid genome [11]. By *Eco*RI cleavage and analysis of resultant fragments it was suggested that they are also arranged in tandem repeats [12]. Here we describe the cleavage sites of restriction enzymes *Hin*dIII, *Eco*RI and *Bam*HI and the size of the repeat unit.

## 2. Materials and methods

### 2.1. *Isolation of DNA*

The head and the extremities of day 12 chicken embryos were cut off, the intestines were removed and the trunks immediately frozen in liquid nitrogen. The DNA was isolated by the proteinase K procedure as in [13] with subsequent degradation of RNA by incubation with RNase and a final phenol extraction. The average molecular weight of this DNA was $\sim 150–200 \times 10^6$.

### 2.2. *Fragmentation by restriction enzymes*

DNA (25 µg) was digested at 37°C for 90 min in 100 µl final vol. by 100 U of each of the restriction enzymes. *Eco*RI (Miles Research Products) was used in a buffer containing 0.1 M Tris (pH 7.5), 0.05 M NaCl, 0.01 M $MgCl_2$. Digestion reactions with *Hin*dIII (Boehringer, Mannheim) contained 0.01 M Tris (pH 7.6), 0.05 M NaCl, 0.01 M $MgCl_2$, 0.014 M dithiothreitol (Serva) and 0.1 mg/ml bovine serum albumin. *Bam*HI (Boehringer, Mannheim) was used in a buffer containing 0.006 M Tris (pH 7.5), 0.02 M KCl, 0.006 M $MgCl_2$ and 0.006 M dithiothreitol. Digestion reactions with *Eco*RI and *Hin*dIII were stopped by addition of 10 µl 25% glycerol, 0.025% bromphenol blue and 5% sodium dodecyl sulfate and incubation was continued for 10 min at 65°C. The same mixture without sodium dodecylsulphate was added to stop *Bam*HI reactions.

### 2.3. *Agarose slab-gel electrophoresis of DNA*

Agarose (Seakem, Rockland, Maine) slab gels (0.5% in 0.03 M Tris (pH 7.6), 0.03 M $NaH_2PO_4$ and 0.001 M EDTA [14]) of 15 × 15 × 0.3 cm were used and electrophoresis was performed at 30 V and 4°C for 16 h. Coelectrophoresis of digestion products from λ-DNA with *Eco*RI and *Hin*dIII and undigested λ-DNA served as references for the determination of the molecular weights of the chicken embryo DNA restriction fragments. After staining with 0.5 µg/ml ethidium bromide in electrophoresis-buffer the gels

were laid on an ultraviolet transilluminator and photographed.

2.4. *Preparation of* $^{125}$*I-labelled rRNA*

Ribosomal RNA was isolated from chicken embryos and iodinated as in [15]. The specific radioactivities of the RNA samples were ~5–10 × $10^6$ dpm/μg.

2.5. *Transfer of the DNA to nitrocellulose filters, hybridization and fluorography*

To transfer the denatured DNA to cellulose nitrate filters (Millipore SA; 0.45 μm) we used the original technique in [16]. After 16 h transfer the filters were shortly immersed in 2 × SSC (0.3 M NaCl, 0.3 M Na-citrate) and baked at 80°C for 2 h in a vacuum oven. Hybridization was performed in 50 ml 2 × SSC containing 50 μg $^{125}$I-labelled 18 S or 28 S rRNA for 20 h at 65°C. Hybridization mixtures with $^{125}$I-labelled 18 S rRNA contained an excess of unlabelled 28 S RNA and vice versa. After washing 2 times in 2 × SSC at 20°C for 20 min RNase A was added (1 mg/100 ml 2 × SSC). Incubation was for 20 min at 37°C followed by another 2 washes in 2 × SSC. After drying the filter strips for 60 min at 60°C in a vacuum oven they were dipped through a solution of 7 g PPO (Merck) in diethylether, dried in air and for fluorography [17] placed tightly against Kodak X-OMAT R film at −70°C for various times.

### 3. Results and discussion

DNA of chicken embryos was cut by restriction enzymes *Eco*RI, *Hin*dIII or *Bam*HI. Figure 1a–c shows the fluorography patterns obtained after cleavage of DNA with each of the three enzymes and hybridization either with $^{125}$I-labelled 18 S or 28 S rRNA. The molecular weights of the fragments were calculated by a comparison to *Eco*RI and *Hin*dIII fragments of λ-DNA, the molecular weights of which are known [18,19]. In the case of *Eco*RI cleavage two fragments were observed. The larger one has mol. wt 15.05 × $10^6$ and hybridizes to a very large extent to 18 S rRNA. After longer exposure during fluorography there was also a faint band when hybridizing with 28 S rRNA (not shown here). The smaller fragment which has mol. wt 5.25 × $10^6$ hybridizes also with 18 S rRNA but to a larger extent with 28 S rRNA. The molecular weights of these *Eco*RI fragments roughly correspond to those reported in [12] (5 × $10^6$ and 12–14 × $10^6$) but hybridization of 28 S rRNA to the large fragment could not be detected [12]. This discrepancy could be explained by small amounts of labelled 18 S rRNA contaminating our labelled 28 S rRNA preparation. However, the RNA purification procedure involved centrifugation on denaturing formamide sucrose gradient [15] and an analysis of the 28 S rRNA by polyacrylamide gel electrophoresis gave no evidence for such a contamination. Moreover, excess unlabelled 18 S rRNA was present in the hybridization mixture. This mixture was also used for hybridization with DNA fragments obtained by cleavage with other restriction enzymes and no contamination was apparent. Digestion with *Hin*dIII results in only one fragment with mol. wt 20.3 × $10^6$ which hybridizes as well to 18 S rRNA as to 28 S rRNA. Restriction with *Bam*HI results in a fragment of mol. wt 3.8 × $10^6$ visible with 18 S rRNA as well with 28 S rRNA and two additional fragments of mol. wt 3.4 × $10^6$ and 1.2 × $10^6$ which can only be detected with 28 S

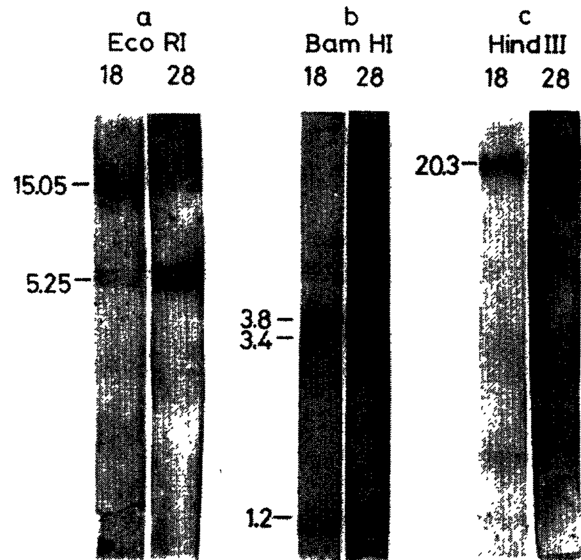


Fig.1. Fluorography of chicken embryo rDNA restriction fragments obtained by cleavage with *Eco*RI (a), *Bam*HI (b) or *Hin*dIII (c) and hybridization with $^{125}$I-labelled 18 S or 28 S rRNA.

rRNA. The molecular weights of the fragments obtained after cleavage with different enzymes and the relative intensities of the bands after fluorography are summarized in table 1. As the sum of molecular weights of the *Eco*RI fragments corresponds to that of the *Hin*dIII fragment, and the large *Eco*RI fragment covers part of the 18 S rRNA and of the 28 S rRNA genes, we conclude that the repeating unit has mol. wt $20.3 \times 10^6$ which is identical to the size of the *Hin*dIII fragment. Moreover, by the relative intensities of the bands after fluorography the cleavage sites of *Eco*RI and *Bam*HI can roughly be determined as shown in fig.3. The size of the repeat unit of chicken rDNA resembles that of rRNA from man (mol. wt $20 \times 10^6$ [9]) or mouse (mol. wt $26 \times 10^6$ [9,10]) which are definitely larger than that of *Xenopus laevis* (mol. wt $6.8-10.5 \times 10^6$ [2]). In addition we did not obtain any evidence for such a marked length heterogeneity as found in *Xenopus laevis* [1–3].

A more precise investigation on the location of the cleavage sites was performed by degradation of DNA with a combination of each of two of the enzymes used. Figure 2a shows the pattern of fragments after cleavage of chicken embryo DNA with *Eco*RI and *Bam*HI. The large *Bam*HI fragment (mol. wt $3.8 \times 10^6$) had been cut into two smaller fragments. The larger one can be detected after hybridization to 18 S rRNA as well to 28 S rRNA and has mol. wt $2.9 \times 10^6$. The smaller one with mol. wt $0.9 \times 10^6$ is only visible after hybridization to 18 S rRNA. Another fragment with mol. wt $1.2 \times 10^6$ is found after hybridization to

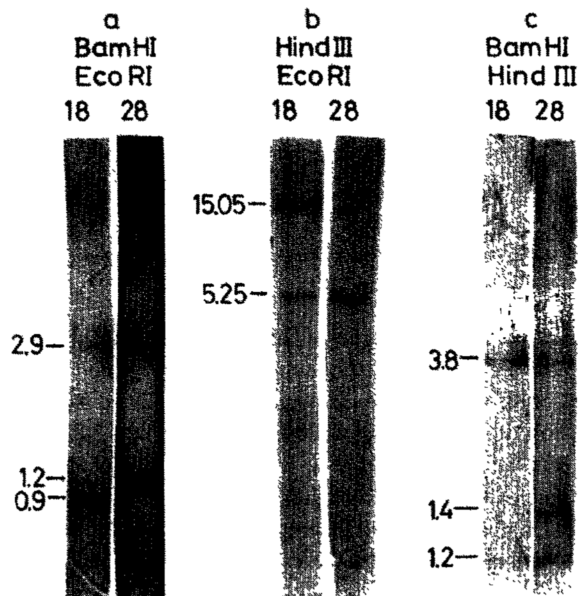


Fig.2. Fluorography of chicken embryo rDNA restriction fragments obtained after cleavage with *Bam*HI/*Eco*RI (a), *Hin*dIII/*Eco*RI (b) or *Bam*HI/*Hin*dIII (c) and hybridization with $^{125}$I-labelled 18 S or 28 S rRNA. Electrophoretic mobilities of the reference samples (λ restriction fragments) were different in all 3 experiments.

28 S rRNA. Because of the relative high intensity of the band after fluorography, we draw the conclusion that this band represents two different fragments of the same size. The first is identical to the original *Bam*HI fragment of mol. wt $1.2 \times 10^6$ and the second results by an *Eco*RI cleavage of the other original *Bam*HI fragment of mol. wt $3.4 \times 10^6$. This experiment substantiates the suggested cleavage sites of *Eco*RI and *Bam*HI as shown in fig.3.

Figure 2b shows the degradation of chicken embryo DNA by *Eco*RI and *Hin*dIII. There is no apparent change of the size of *Eco*RI fragments as compared to a degradation by *Eco*RI alone, but we could not detect a hybridization of 28 S rRNA to the larger fragment even after a very long exposure during fluorography. This can only be interpreted by a *Hin*dIII cleavage site near the 28 S rRNA gene within the long untranscribed spacer (as shown in fig.3). Obviously the size reduction of the large *Eco*RI fragment is too small to be detected by slab gel electrophoresis under the conditions used. A degra-

Table 1
Chicken embryo rDNA fragments obtained by cleavage with different restriction endonucleases

| Restriction enzyme | Mol. wt ($\times 10^{-6}$)[a] | Relative intensities of fluorography bands after hybridization with | |
|---|---|---|---|
| | | 18 S | 28 S |
| *Eco*RI | 15.05 ± 0.3 | 70% | 5% |
| | 5.25 ± 0.2 | 30% | 95% |
| *Hin*dIII | 20.3 ± 0.4 | 100% | 100% |
| *Bam*HI | 3.8 ± 0.1 | 100% | 33% |
| | 3.4 ± 0.1 | – | 33% |
| | 1.2 ± 0.1 | – | 33% |

[a] Average values from 8 experiments

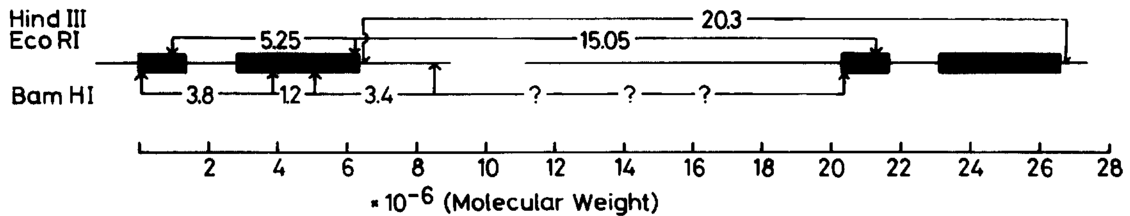


Fig.3. Arrangement of chicken embryo rDNA and cleavage sites of *Eco*RI, *Bam*HI and *Hin*dIII.

dation with a mixture of the enzymes *Bam*HI and *Hin*dIII (fig.2c) further supports the suggested location of the *Hin*dIII cleavage site. There is no degradation of the large *Bam*HI fragment (mol. wt $3.8 \times 10^6$) but the fragment of mol. wt $3.4 \times 10^6$ which covers part of the 28 S rRNA gene and part of the untranscribed spacer is cut leaving a fragment of $1.4 \times 10^6$, which is in agreement with the cleavage site of *Hin*dIII, indicated above.

A comparison of these cleavage sites to those in *Xenopus laevis* [2] shows a very close relationship, which is consistent with a high degree of conservation of rRNA gene sequences during evolution to amphibia and birds. There seems also to be a conservation of *Eco*RI and *Bam*HI cleavage sites at least within the rRNA coding DNA sequence during evolution to higher eukaryotes like mouse or man. On the other hand, a *Hin*dIII cleavage site in these organisms is found within the 18 S rRNA gene [9,10] and not beside the 28 S rRNA gene as in chicken.

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