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DNase I hypersensitive sites of the chromatin for Drosophila melanogaster ribosomal protein 49 gene

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ABSTRACT

By using an indirect end-labelling technique for mapping, five DNase I hypersensitive sites have been located in *Drosophila melanogaster* chromatin at the 5'-end of the gene coding for ribosomal protein 49. These sites typically span about 100-160 base pairs and are approximately the length of a nucleosome apart (center to center distance ca 245 bp). This is the first analysis of the chromatin structure of a constitutive house-keeping gene. The results support the hypothesis that the presence of such a DNase 1 hypersensitive site in chromatin is necessary for transcription *in vivo*. The presence of such sites may reflect some local changes in the conformation of the chromatin in the presumptive regulatory region.

INTRODUCTION

The use of DNase I as a probe of chromatin structure has provided information which appears relevant to the understanding of gene regulation. DNase I hypersensitive sites (or short regions) have been observed in the chromatin of several organisms [*Drosophila* (1-4), chicken (5,6,7), rat (8)], and of the virus SV40 (9-12)). In most cases such sites are found at the 5'-end of genes which are being or can be expressed in the cell type under analysis. Examples to date include the hsp 70 and hsp 83 genes (2), hsp 28, hsp 23, hsp 26, and hsp 22 genes (3), and the histones genes (4) in *Drosophila*, the α-globin genes (7), the embryonic β-globin gene, the RAV-0 eV-3 locus, and the integrated adenovirus genes in chicken (5), the preproinsulin II gene in rat (8), and the late genes of SV40 (9-12). That such DNase I hypersensitive sites are tissue specific (found only in chromatin of cells which can express the gene) has been demonstrated for the α-globin gene (7) and β-globin genes (5,13) in chicken and for the preproinsulin II gene (8) in

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rat.

The gene we have studied is the *Drosophila* gene for ribosomal protein 49, a constitutive house-keeping gene (14). This single-copy gene codes for a ribosomal protein of approximately 20,000 daltons. It is apparently transcribed at a low rate in all cells. A 17.7 kb DNA segment, including this gene, was cloned into a Charon 4 phage, c25 (14). The various recombinant plasmids used in this investigation were derived from restriction fragments of the parent phage.

Five DNase I hypersensitive sites in chromatin were found at the 5'-end of the gene by using the indirect end-labelling technique for mapping (2). These sites typically spanned about 100-160 bp and were approximately the length of a nucleosome apart (center to center distance). Since no corresponding sites were found in the DNA of plasmid H4.0, which contained the gene and its 5'-flanking sequences, we conclude that the presence of these DNase I hypersensitive sites is a property of the chromatin structure.

MATERIALS AND METHODS

Purification of Nuclei from Drosophila Embryos

Nuclei were isolated and purified from 6-18 hour old *Drosophila melanogaster* Oregon R embryos as described previously (3). The final concentration of nuclei was adjusted to 5 X 10^8/ml in DNase I digestion buffer (60mM KCl/15 mM NaCl/15 mM Tris-HCl, pH 7.4/ 0.5 mM DTT/ 0.25 M sucrose/ 0.5 mM $CaCl_2$ / 3 mM $MgCl_2$) for subsequent digestion.

DNase I Digestion of Chromatin

Aliquots of 500 µl of nuclei, suspended in DNase I digestion buffer, were digested with various concentrations of DNase I (3.75-15 units/ml, final concentration) for 3 minutes at 25°C. The reaction was stopped by adding Na_2EDTA to a final concentration of 12.5 mM, followed by adding SDS to 0.5% (1).

Purification of DNA

DNA was purified from nuclei as described previously (1).

Secondary Restriction Digestion

Secondary restriction digestions were carried out under the conditions suggested by the manufacturer (Bethesda Research

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Laboratories) and terminated by phenol extraction (phenol / chloroform / isoamyl alcolol / hydroxy quinoline=50:50:1/0.1%(v:v:v/wt/v)). The DNA was purified by ethanol precipitation (1).

Extraction and Purification of High Molecular Weight Embryo DNA

Nuclei were isolated from embryos, as above, and were immediately lysed in extraction buffer (2% SDS/7M urea/0.35 M NaCl/1 mM Na_2EDTA/10 mM Tris base). DNA was purified as described previously (1).

DNase I Digestion of Genomic DNA and Plasmid DNA

The conditions used were the same as those used for the digestion of chromatin, except that the concentrations of DNase I used for genomic DNA were from 0.0025 units/ml to 0.02 units/ml, while those for plasmid DNA were from 0.004 units/ml to 0.032 units/ml.

Analysis of DNA Fragments from Chromatin Digestion and Genomic DNA Digestion

Gel electrophoresis of the DNA samples and Southern transfer to nitrocellulose were performed as described (1), except that a uniform sample size of 10μg of DNA was loaded per lane in all cases. Plasmid probes were labelled in vitro by nick translation as described (1) except that the DNA concentration was 1 μg/ml. The final concentration of DNase I was 0.0012 units/ml and the incubation temperature was 12.5°C. Hybridization of the filter and autoradiography were carried out as described before (1).

Filling-in of the Staggered Ends of Linearized Plasmid with Radioactive Nucleotides

The plasmid H4.0 was linearized by cutting with the restriction enzyme Bam HI. The staggered ends generated were filled in with radioactive nucleotides. The reaction mixture contained 10 mM $MgCl_2$, 10 mM Tris-HCL (pH 7.4), 10 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, 2 μg of linearized plasmid, 5 units of E. coli DNA polymerase I--Klenow fragment (Boehringer) and 1.5 μM ^{32}P-dNTP's (~500 Ci/mmole) in a final volume of 100 λ(15). The reaction was carried out at 37°C (1 hour) and was terminated by phenol extraction. The labelled plasmid fragments were purified using Sephadex G-50

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chromatography.

Analysis of DNA Fragments from Plasmid H4.0

Gel electrphoresis was carried out as before except that a sample size of 0.2 μg of DNA per lane was used. After electrophoresis, the gel was dried and autoradiography was carried out using Kodak XR film with a DuPont Lightning-Plus intensifying screen.

Plasmid Subclones

Subclones were generated from phage c25 (14) using the plasmid pBR 322 (16). Ligation, transformation and selection of recombinant plasmids were carried out as described by Barnett *et al.*(17). Plasmid DNA (18) and phage DNA (19) were prepared according to standard procedures.

Northern Gels

2.0μg of poly $(A)^+$ RNA were separated on 1.5% agarose CH_3HgOH gels. The protocol used for electrophoresis and for treatment of the gels, preparation of paper, RNA transfer, and hybridization was that described by Alwine, *et al*. (20). Labelling of H4.0 DNA by nick translation for hybridization was as previously described (14).

Formation of R-loops and Electron Microscopy

R-loop formation, treatment and spreading of the DNA-RNA hybrids were performed as previously described (21) except that the DNA was trioxsalen crosslinked before hybridization (22).

S1 Nuclease Protection Mapping

3'-end labelled DNA (0.05 μg) was hybridized with cytoplasmic poly $(A)^+$ RNA (10 μg) in a total volume of 30λ of 70% formamide, 0.4 M NaCl, 1 mM EDTA, 0.05 M Pipes (pH 6.8), and 0.01 M EDTA at 56° for 3 hours after treatment at 80° for 2.5 minutes. The incubation mixture was rapidly cooled, diluted 10 fold into S_1 buffer [30 mM NaOAc (pH 4.5), 0.02 M $ZnSO_4$, 0.2 M NaCl] and treated with 30 units of S_1 nuclease (BRL) for 30 minutes at 37° (23). The hybrids were separated on 4.5% acylamide gels in 8 M urea, 900 mM Tris, 900 mM borate, 25 mM EDTA (pH 8.3) (24).

Biohazard Considerations

All recombinant DNA containing strains and phage were propaged under NIH P1-EKI containment conditions.

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RESULTS

A restriction map of the region of the *Drosophila* genome containing the gene for ribosomal protein 49 and a gene of unknown function is present in Figure 1. H4.0, HS0.3 and HR0.6 are recombinant plasmids containing the corresponding restriction fragments as shown. The H4.0 recombinant plasmid contains sequences complimentary to two *Drosophila* poly $(A)^+$ RNA's. These RNA's were sized and localized on H4.0 DNA using Northern hybridization (20) and R-looping techniques (21). The results are shown in Figure 2.

An indirect end-labelling technique (2) was used to map the DNase I hypersensitive sites in the chromatin of this region. Chromatin (in the intact nuclei) was lightly digested with DNase I. The DNA was then purified and cleaved wth the restriction enzyme Hind III. The DNA fragments were run out on an agarose gel, a Southern blot prepared, and the filter hybridized using HR0.6 (the 0.6 kb Eco R1-Hind III restriction fragment) as the probe. By autoradiography one then observes the set of DNA fragments bounded at one end by the right hand Hind III site, and at the other end, by a DNase I hypersensitive site or the left hand Hind III site. Five DNase 1 hypersensitive sites were observed, at 1.58 +/- 0.07, 1.36 +/- 0.08, 1.10 +/- 0.08, 0.82 +/- 0.07, and 0.62 +/- 0.05 kb from the right hand Hind III site (see lanes A, B and C of Figure 3).

When similar experiments were carried out using genomic DNA

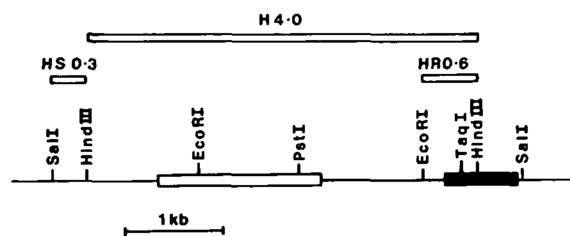

Figure 1. Restriction Map of the Cloned Region Containing the Gene for Ribosomal Protein 49 and a Gene of Unknown Function.

The filled-in block represents the ribosomal protein 49 gene, the clear block the gene of unknown function. Subclones derived from the original Charon phage c25 (12) used as probes in these experiments are shown above the map.

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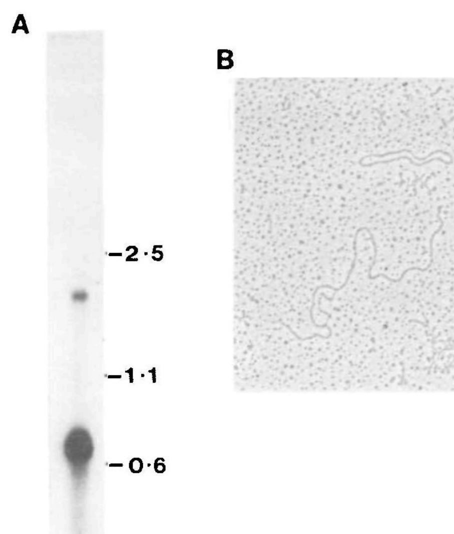

Figure 2. Transcripts of Subclone H4.0 Genes.

A. Northern blot of cytoplasmic Poly $(A)^+$ RNA's complimentary to H4.0. The 1.7 kb minor transcript corresponds to the gene of unknown function. The 0.65 kb transcript is the mRNA for ribosomal protein 49. An adjacent Hind III subclone, from c25, also contains sequences that hybridize to the ribosomal protein 49 transcript (data not shown). This indicates that this gene spans the Hind III site as shown in Figure 1.

B. Electron mircograph of R-looped H4.0 DNA. The DNA was linearized with the restriction enzyme Sal I and hybridized under conditions favoring R-loop formation. From left to right, along the molecule, are the minor 1.7 kb transcript and the truncated ribosomal protein 49 gene. Beyond this lies the bulk of the pBR322 vector DNA. Pictured above the linear molecule is a circular pBR322 size marker (4361 bp).

which had been digested with DNase I to an equivalent extent, very faint bands might be detected at the same positions (see lanes E, F and G of Figure 3). Given our standard method of purification of genomic DNA from nuclei, a low level of digestion of the DNA in chromatin by endogenous nucleases prior to isolation is not impossible. In fact, Hewish and Burgoyne have used endogenous nucleases to study chromatin structure in rat liver (25). Alternatively, these faint bands could indicate a preference of DNase I for specific DNA sequences.

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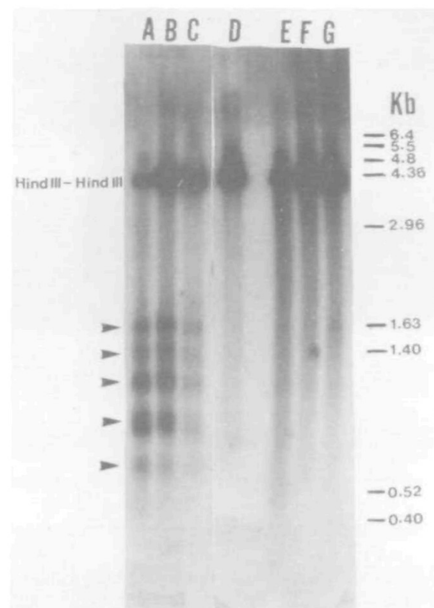

Figure 3. Autoradiogram Showing DNA Fragments Bounded by a DNase I Hypersensitive Site and the Right Hand Hind III Site.

Lanes A, B, and C contain DNA fragments generated by a decreasing amount of DNase I (15, 7.5 and 3.75 units/ml, respectively) followed by digestion of the DNA fragments with Hind III.

Lanes E, F, and G contain DNA fragments generated by digestion of genomic DNA with decreasing amounts of DNase I (0.02, 0.005, and 0.0025 units/ml, respectively) followed by digestion of the DNA fragments wth Hind III.

Lane D contains DNA fragments from genomic DNA digested with Hind III alone.

Horizontal arrows indicate DNA fragments bounded by a DNase I hypersensitive site and the right hand Hind III site.

To determine whether or not these sites were chromatin specific, the following experiment was carried out. H4.0, the recombinant plasmid made up of pBR 322 and the Hind III-Hind III restriction fragment (see Figure 4), was linearized using the restriction enzyme Bam HI. The staggered ends were filled with ^{32}P-dNTP's by E. coli DNA polymerase I-Klenow fragment. A second restriction cut was made using Sal I, generating two labelled fragments of 8.1 kb and 275 bp. These fragments were

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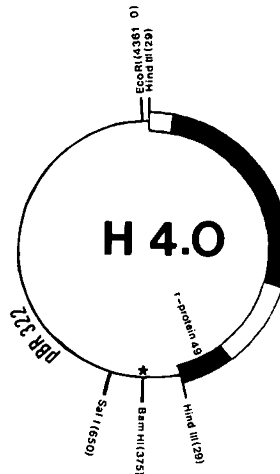

Figure 4. The Recombinant Plasmid H4.0.
The thin line represents pBR 322 DNA sequence in H4.0, while the thick line represents the *Drosophila* insert (Hind III-Hind III fragment of 4.0 kb). The solid regions represent the gene for ribosonal protein 49 and the adjacent gene. The numbers in parenthesis are the cleavage coordinates for restriction enzyme sites in pBR 322 (26). The asterisk represents the label of ^{32}P-dNTP's.

then digested with different concentrations of DNase I. After purification of the DNA, the fragments were run on a 1.3% TAE agarose gel. The gel was dried down and autoradiography was performed. Any band that was shorter than 8.1 kb and longer than 275 must be generated by the DNase I digestion. No distinct bands, but a smear was seen, indicating that there are no DNase I preferential cutting sites in this DNA fragment per se (see Figure 5).

Similar mapping of the chromatin-generated sites was carried out using HS 0.3 (which contains the 0.3 kb Hind III-Sal I restriction fragment) as a probe for the segment of the genome bounded by the two Sal I sites (see Figure 1). Five bands were observed at 2.70, 2.95, 3.20, 3.50 and 3.70 kb from the left Sal I site (Figure 6). Note that the band of highest molecular weight is relatively faint, suggesting that the corresponding DNase I hypersensitive site may be less sensitive than the

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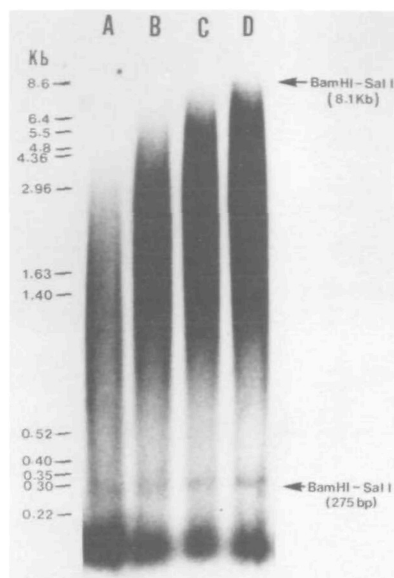

Figure 5. DNAse I Digestion of End-Labeled Bam H1-Sal I Restriction Fragments of H4.0.
0.032, 0.016, 0.008 and 0.004 units/ml of DNase I were used to generate the samples shown in lanes A, B, C, and D, respectively. The horizontal arrows indicate the two parental Bam H1-Sal I restriction fragments.

others. In figure 3, which shows DNA fragments bounded at one end by the right Hind III site and at the other end by the same DNase I hypersensitive sites, the smallest band is also fainter than the others. This lends further support to the above suggestion. Examination of a 9.6 kb region, bounded by Xba I sites, indicated that there are no DNase I hypersensitive sites within 1.4 kb of the other end of the gene encoding ribosomal protein 49 (data not shown).

When these DNase I hypersensitive sites were placed on the map, the mid-points of these sites were approximately at 260, 460, 750, 1010, and 1240 bp from one end of the gene for ribosomal protein 49. The left-most site was actually very close to or within the adjacent gene (see Figure 8). To determine the orientation of transcription of the ribosomal

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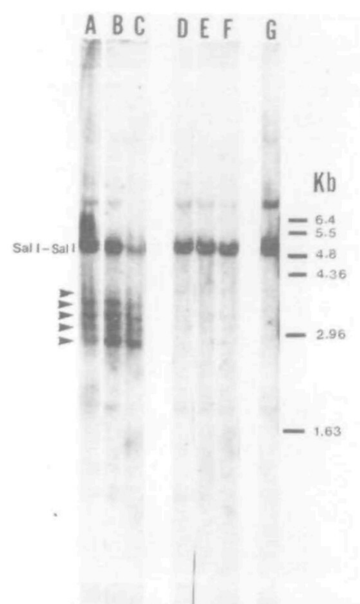

Figure 6. Autoradiogram Showing DNA Fragments Bounded by a DNase I Hypersensitive Site and the left hand Sal I Site.

Lanes A, B, and C contain DNA fragments generated by digestion of chromatin with decreasing amounts of DNase I (15, 7.5, and 3.75 units/ml, respectively) followed by digestion of the DNA fragments with Sal I.

Lanes D, E, and F contain DNA fragments generated by digestion of genomic DNA with decreasing amounts of DNase I (0.02, 0.005 and 0.0025 units/ml, respectively) followed by digestion of the DNA fragments with Sal I.

Lane G contains DNA fragments generated from genomic DNA by digestion with Sal I alone.

Horizontal arrows indicate DNA fragments bounded by a DNase I hypersensitive site and the left hand Sal I site.

protein 49 gene, an S_1 nuclease protection experiment was carried out (see Figure 7). The recombinant plasmid HR0.6 DNA was cut wth the restriction enzyme Taq I, and the appropriate fragments were isolated and labelled at the 3'-end as described above. The fragment protected indicated the direction of transcription to be from left to right for the map given in Figure 8. This places the chromatin DNase I hypersensitive sites at the 5'-end of the gene.

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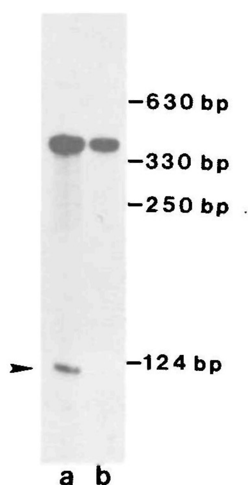

Figure 7. S_1 Protection Experiment to Determine the Direction of Transcription of Ribosomal Protein 49 Gene.

HR0.6 was digested with the restriction enzyme Taq I. These Taq I fragments were 3'-end labelled and the fragment containing the ribosomal protein 49 gene Hind III site was isolated. The labelled DNA was denatured and hybridized with poly $(A)^+$ RNA under conditions favorable for hybrid formation. These hybrids were challanged with S_1 nuclease and the digestion products were analysed by gel electrophoresis.

In lane A, one observes a 125 bp protected fragment (arrow). No protected fragment was evident when the DNA was renatured in the absence of poly $(A)^+$ RNA, as shown in lane B. The upper bands represent renatured, 3'-end labelled DNA.

DISCUSSION

DNase I hypersensitive sites have been found in chromatin at or near the 5'-end of a number of genes in different systems [heat shock protein genes (2,3) and histone genes (4) in *Drosophila*, globin genes in chicken (5,7), preproinsulin II gene in rat (8) and late genes of SV40 (9-12)]. Since this chromatin configuration is not limited to active genes, but is also found at genes that have the potential to be active (as in the case for the heat shock genes), one may speculate that the local conformational changes in chromatin structure at the 5'-end of genes as revealed by local increased susceptibility to DNase I is a necessary but not sufficient condition for gene activation. The tissue specificity of the pattern of DNase I hypersensitive

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Figure 8. Correlation of DNase I Hypersensitive Sites with Regions of Transcription.

The filled-in block represents the gene for ribosomal protein 49, the clear block is the gene of unknown function.

The upper vertical arrows indicate the positions of the mid-points of the DNase I hypersensitive sites obtained from mapping experiments (using the right hand Hind III site as reference point). The lower vertical arrows indicate the postitions of the mid-points of such sites obtained from mapping experiments (using the left Sal I site as reference point).

The horizontal arrow indicates the direction of transcription.

sites, as demonstrated for the α-globin gene (7) and β-globin genes in chicken (5,13) and the preproinsulin II gene in rat (8), further suggests that there is a positive correlation between this chromatin structure and gene expression.

The conformational changes in chromatin that are being detected may be at the level of individual nucleosomes, at the level of higher order structure, or both. One infers that this change results in a chromatin conformation which permits, but does not compel, gene expression.

Since the 5'-end of the ribosomal protein 49 gene is only about 1.3 kb from the end of the adjacent gene, the most distal of the five hypersensitive sites from the 5'-end of the ribosomal protein 49 gene is either very close to or within this transcript. (The precise end points of this gene are not known.) The possibility that some of these sites may be pertinant to the expression of this adjacent gene should not be ignored.

Multiple DNase I hypersensitive sites are not unique to the ribosomal protein 49 gene. Another example is the hsp 83 gene (2) in _Drosophila_. In _Drosophila_, there are 70-80 ribosomal proteins. These proteins are present in constant ratios in the ribosomes and in the cells as well. In

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addition, the amount of ribosomal protein present is regulated coordinately with the synthesis of rRNA (27). It is tempting to speculate that the requirements of such regulation may necessitate a long stretch of DNA as a regulatory element. Perhaps as a consequence, multiple DNase I hypersensitive sites are found.

The periodicity of 200-290 bp for the five DNase I hypersensitive sites may be of some importance. A similar spacing of sites is observed at the 5'-ends of hsp 83 gene (2). One possible suggestion is that there may be similar structural changes in each nucleosome in the presumptive regulatory region and that these changes are recognized by DNase I. Hence cuts are made at each nucleosome or linker and a periodicity of cutting sites, of roughly the length of a nucleosome, is generated.

Future research focussing on the molecular structure of the 5'-DNase I hypersensitive sites may give us some insights into the problem of regulation of gene expression.

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