The EMBO Journal vol.3 no.7 pp.1635 – 1641, 1984

Chromatin structure of a hyperactive secretory protein gene (in Balbiani ring 2) of *Chironomus*

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Communicated by J.-E.Edstrom

We examined the chromatin structure of a Balbiani ring (secretory protein gene) in the salivary glands of *Chironomus* larvae in its hyperactive state after stimulation with pilocarpine. For the inactive state of the gene an established tissue culture cell line, not expressing the gene, was used. Electron microscopy showed an RNA polymerase density of ~38/μm. Micrococcal nuclease digestion of purified nuclei followed by DNA transfer and hybridization revealed a smear with no recognizable discrete DNA fragments. Without pilocarpine stimulation a faint nucleosomal repeat was superimposed upon the smear, and in tissue culture cells a clear nucleosomal repeat was revealed. The restriction enzyme *Xba*I, which has a 6-bp recognition sequence, cut the gene in the hyperactive chromatin state, but not in its inactive conformation. The combined results are best explained by the absence of most of the nucleosomes in this hyperactive RNA polymerase II transcribed gene.

Key words: Chironomus/Balbiani ring/secretory protein gene/chromatin structure

Introduction

Bulk chromatin, considered to be transcriptionally inactive, is built up of nucleosomes (for review, see Igo-Kemenes *et al.*, 1982). The controversy concerning the presence or absence of nucleosomes in transcribing chromatin (see *Cold Spring Harbor Symp. Quant. Biol.*, **42**, 1978) has still not been solved (e.g., Levy and Noll, 1981; Bellard *et al.*, 1982; Sargan and Butterworth, 1982; Smith *et al.*, 1983; Lohr, 1983; Mathis *et al.*, 1980; Weisbrod, 1982). In particular the question remains open whether structural proteins like histones or modified histones are present during transcription or not. The chromatin of ribosomal transcription units saturated with RNA polymerases seen in the electron microscope is extended to a contour length approximately equivalent to B-DNA (Laird *et al.*, 1976; McKnight and Miller, 1976; Trendelenburg *et al.*, 1976). On the other hand, chromatin of non-ribosomal loci exhibiting intermediate or sparse RNA polymerase frequencies is distinctly beaded. The density of beads, considered to be nucleosomes, decreases with increasing number of ribonucleoprotein (RNP) fibrils (McKnight *et al.*, 1978; Scheer, 1978). A difficulty with biochemical experiments is that the proportion of cells transcribing a particular sequence at a given moment and the rate of transcription are usually unknown and may frequently be quite low (Lilley, 1978). If non-expressed pseudogenes are present, the analysis of transcribed genes becomes even more complicated. Moreover, a two-step process of gene activation has been postulated, based on analysis of puffing in polytene chromosomes, the first step being independent of transcription (Lezzi, 1982). Since we are interested in the chromatin structure at the site of transcription, we decided to study gene systems which are very active in transcription. The results obtained with nucleoli of growing *Dictyostelium discoideum* (Ness *et al.*, 1983; Labhart *et al.*, 1983) suggest that nucleosomes were absent from the coding sequence. They are compatible with a model deduced from observations made on nucleolar chromatin in *Xenopus laevis* oocytes (Labhart and Koller, 1982; Labhart *et al.*, 1983) that most of the histones might be absent during transcription. It would thus be interesting to study also a hyperactive gene transcribed by RNA polymerase II. In salivary glands of *Chironomus* larvae the transcription in the Balbiani ring 2 (BR 2) genes is stimulated by the treatment of animals with pilocarpine (Mähr *et al.*, 1980). In addition, intense transcription of the BR 2 has been visualized by electron microscopy even in the absence of pilocarpine stimulation (Lamb and Daneholt, 1979). Therefore this gene system appears to be ideal as a model to study the structure of the chromatin actively transcribed by RNA polymerase II.

Results

Electron microscopy

Chromosomes IV manually isolated from pilocarpine-stimulated and non-stimulated larvae were decondensed in 20 μl of 1 mM triethanolamine chloride (pH 9), 0.2 mM EDTA and then prepared for electron microscopy. Thin section studies (for review, see Daneholt *et al.*, 1982) have shown that BR 2 may be recognized among other active chromosome regions by its large area and by its content of special RNP granules. Only BR 1 exhibits similar features (Olins *et al.*, 1982). For unequivocal identification, BR 2 was occasionally isolated from unfixed chromosomes IV by micromanipulation (Frey *et al.*, 1982) followed by centrifugation of several rings onto an electron microscope grid. The transcription units visualized under these conditions were indistinguishable from those considered to belong to the BR 2 in entire chromosomes.

Figures 1A and B show characteristic areas of well-spread transcription units at high magnification. The chromatin axis shows rows of beads which are considered to represent RNA polymerases since most of them had an RNP fibril attached. Since the RNA polymerases were densely packed (Figure 1A), the underlying chromatin fiber could be seen only in rare places, as thin, DNA-like fibers (Figure 1B, large arrows; for comparison purified nicked-circular PM 2 DNA, small arrows). It was never possible to find entire transcription units. The longest fragments found were 8 – 9 μm and in the well-spread preparations the density of putative RNA polymerases was 38 (±2) per μm (35 μm analysed). We also prepared samples from salivary glands of larvae which were not stimulated with pilocarpine. The spreading of these chromosomes was more difficult and less reproducible than those

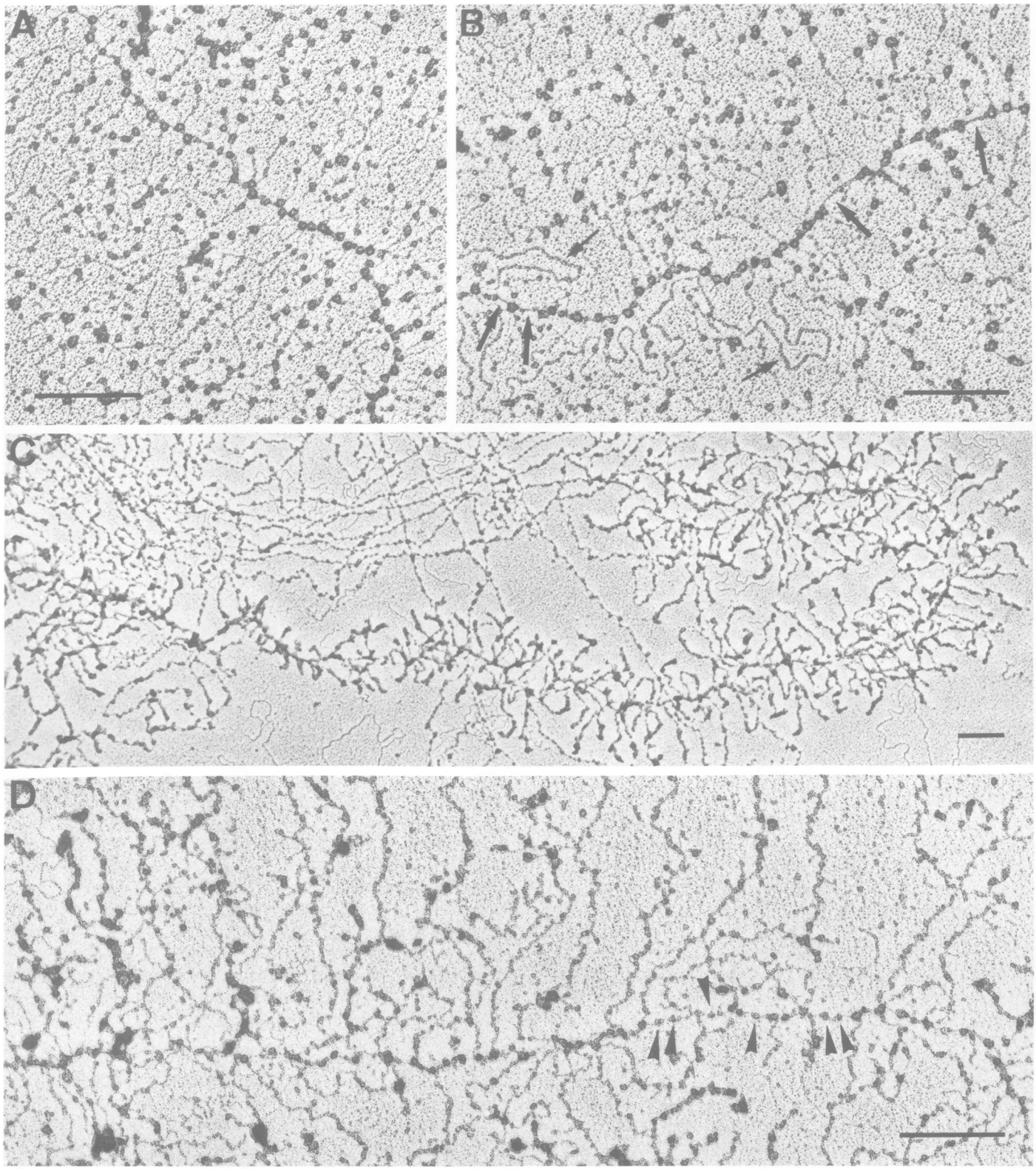

Fig. 1. Electron micrographs of BR 2 transcription units. **A** and **B** after pilocarpine stimulation. **C** and **D** without pilocarpine stimulation. Note in **D** a lower density of RNP fibrils than in **A** and **B**. In **D** occasionally beads are visible (arrowheads) which have no connection to an RNP fibril, opposite to **A** and **B** where most of the beads seem to be connected to RNP fibrils. In **B** the large arrows point to thin DNA-like fibrils connecting putative RNA polymerases. For comparison PM 2-DNA (nicked circular form II) was co-prepared (small arrows in **B**). The bars represent 0.2 μm.

from the stimulated animals because the glands were not emptied as with pilocarpine treatment. Probably the secretory proteins tend to stick to the chromosomes and for this reason the appearance of the transcription units prepared from the non-stimulated animals cannot be strictly compared with those of pilocarpine-stimulated animals. Our observations made on the spreadings of transcription units from non-stimulated animals were the same as described by Lamb and

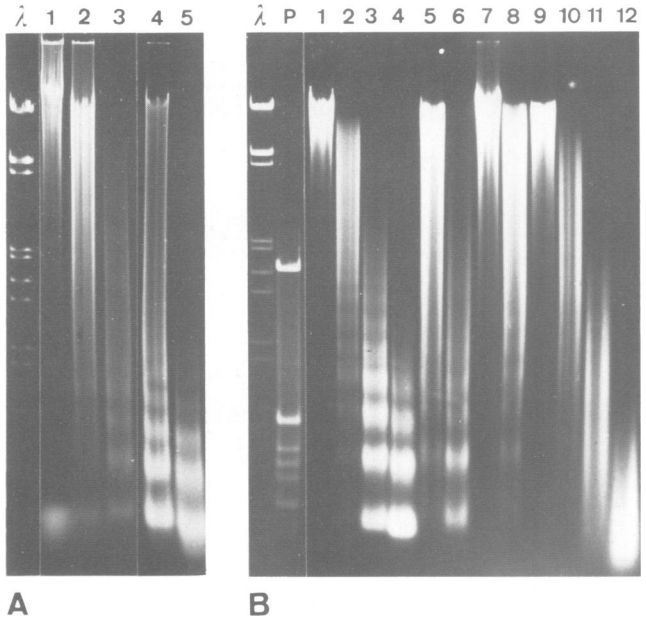

Fig. 2. Gel electrophoretic analysis of the DNA fragments produced from nuclei and DNA samples by digestion with micrococcal nuclease. **(A) 1–3:** nuclei of 450 glands isolated from pilocarpine-stimulated larvae digested with 4 units of enzyme for 1, 4 and 12 min. **4–5**: nuclei of L1-cells digested with 1.5 units of enzyme per A_{260} unit for 1 and 5 min. **(B) 1–4**: nuclei of L1-cells digested with 4.5 units of enzyme per A_{260} unit for 1, 4, 15 and 45 min. **5** and **6**: nuclei of 280 glands from non-stimulated larvae digested with 15 units of enzyme for 5 and 20 min. **7** and **8**: nuclei of 230 glands from pilocarpine-stimulated larvae digested with 15 units of enzyme for 4.5 and 18 min. **9–12:** pure DNA from L1-cells digested with 0.01 units of enzyme/μg for 3, 10, 30 and 90 min. λ: *Eco*RI-*Hind*III digest of λ DNA (wt) with the following sizes: 21 221, 5150, 4974, 4271, 3538, 2024, 1906, 1584, 1375, 947, 832, 564 bp. **P**: *Hinf*I digest of pBR322 DNA with the following sizes: 1631, 517/506, 396, 344, 298, 221/220, 154 bp. The digestions were made at 37°C. Two different batches of micrococcal nuclease were used in the experiments shown in **A** and **B**. The gels were stained with ethidium bromide.

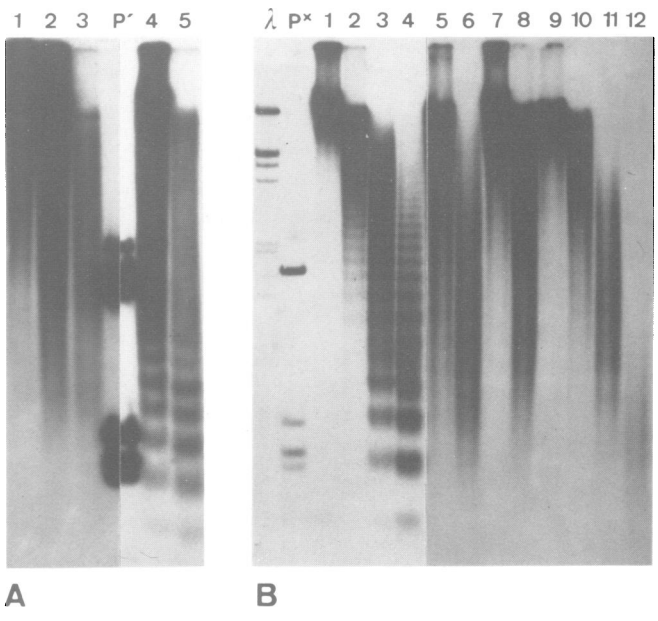

Fig. 3. Micrococcal nuclease digestion of BR 2 chromatin and DNA. Autoradiograms of Southern blots of the gels shown in **Figure 2** after hybridization with plasmid pCp31 **(A)**, or the BR 2 sequence insert of pCp31 **(B)**. **A, 1–5** and **B, 1–12** are as indicated in the legend to Figure 2. **P′** and **P***: bands of *Hinf*I-digested pBR322 hybridized with pCp31, showing the following sizes: 2148 (1631 + 517, only in P′), 1631, 517, 344, 316 bp. The same results were obtained using pCt63 plasmid containing a different sequence of BR 2 gene of *C. tentans* (Sümegi *et al.*, 1982).

Daneholt (1979). Figure 1C shows a representative entire transcription unit and Figure 1D an area of another transcription unit at high magnification. As compared with the findings after pilocarpine stimulation (Figures 1A and B), the well-spread transcription units of non-stimulated animals had a lower RNP-fibril density and occasionally beads were observed which were not connected to RNP-fibrils (arrowheads in Figure 1D).

Digestion of nuclei with micrococcal nuclease

The experiments described were performed with larvae from *C. tentans,* but the plasmid pCp31 used in the hybridization studies contained a BR 2 sequence from the sibling species *C. pallidivittatus* (Jäckle *et al.*, 1982). The 195-bp repeat unit represented by the insert in pCp31 is represented 89 times in *C. pallidivittatus* (Galler *et al.*, 1984) and the homologous unit in *C. tentans* 50–70 times (Wieslander and Lendahl, 1983; Case *et al.*, 1983). The repeat units are tandemly arranged, although not necessarily in one block. We verified the specificity of the hybridization reaction in this not strictly homologous system by *in situ* hybridization. The results are shown and discussed in Materials and methods. Nuclei isolated from salivary glands of pilocarpine-stimulated or not stimulated larvae or from a tissue culture cell line (L1-cells) not expressing the BR 2 gene, were digested with micrococcal nuclease in parallel with deproteinized DNA from L1-cells.

Whereas the deproteinized DNA (Figure 2B, slots 9–12) was degraded into a smear, discrete DNA fragments corresponding to a nucleosomal repeat of ~180 bp were produced in all types of nuclei (Figure 2A, slots 1–5 and 2B, slots 1–8). This repeat size is similar to that which has been found previously (Andersson *et al.*, 1980; Yamaguchi *et al.*, 1982). When the DNA of the gels of Figure 2 was blotted (Southern, 1975), and hybridized with ^{32}P-labelled probes from BR 2 a similar but much sharper pattern with bands representing up to 20 nucleosomes was observed with the DNA extracted from L1 nuclei (Figure 3A, slots 4 and 5, Figure 3B, slots 1–4). The exact repeat size was only determined for the material shown in Figure 3B, slots 1–4, and we found two repeats, one of ~180 bp for the short fragments and one of ~195 bp for the fragments longer than 6-mers. This peculiar chromatin structure will be described in detail elsewhere. However, the DNA from salivary glands of pilocarpine-stimulated larvae revealed a smear (Figure 3A, slots 1–3, Figure 3B, slots 7 and 8). The DNA from glands of non-stimulated larvae also showed mainly a smear, but in addition faint bands corresponding to a nucleosomal repeat with multiples of ~195 bp (Figure 3B, slots 5 and 6). Within the smear from the blot of the deproteinized DNA (Figure 3B, slots 9–12) also weak bands were seen. This repeat is clearly different from that described for the chromatin DNA and it originates most likely from sequence-specific cutting by micrococcal nuclease (Hörz and Altenburger, 1981; Dingwall *et al.*, 1981) within the repeated sequences (Bäumlein *et al.*, 1982; Jäckle *et al.*, 1982; Sümegi *et al.*, 1982) of the BR 2 region.

Comparing Figures 2 and 3 we note that the speed of degradation of the bulk chromatin (ethidium bromide stained gel of Figure 2) is not markedly different from that of the

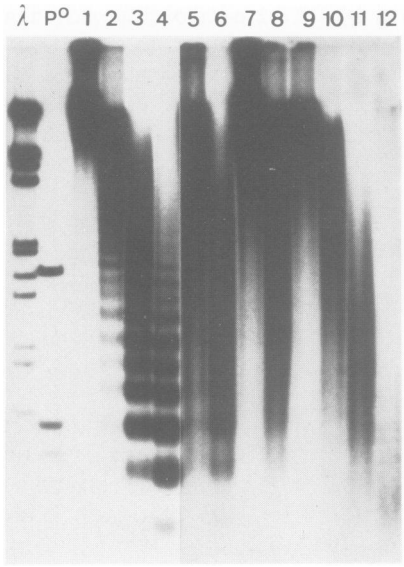

Fig. 4. Micrococcal nuclease digestion of BR 1 chromatin and DNA. Autoradiograms of the filter shown in Figure 3B after re-hybridization with the BR 1 insert of pCt21. **1–12** as indicated in Figure 2B. **P°**: *Hinf*I-digested pBR322 hybridized with pCt21, only bands of 1631, 517/506 bp visible.

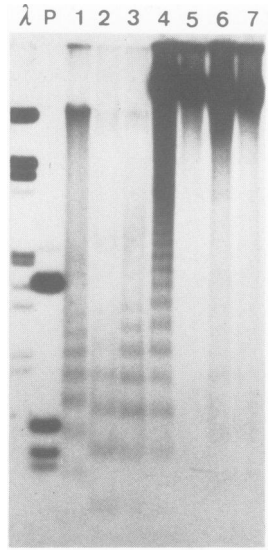

Fig. 5. Digestion of BR 2 chromatin and DNA with *Xba*I. Hybridization with pCp31 on Southern blots of the electrophoretically separated DNA fragments. **(1)** Micrococcal nuclease digest of nuclei from the same L1-cells as used for the *Xba*I digestion (3 units of enzyme per A_{260}, 1 min, 37°C). In this sample the nucleosomal repeat was ~160 bp. **(2** and **3)** *Xba*I-digest of deproteinized DNA from L1-cell nuclei. [6 units **(3)** and 18 units **(2)** of enzyme/150 ng of DNA, 60 min, 37°C]. **(4)** *Xba*I digest of nuclei from salivary glands of pilocarpine-stimulated larvae. (50 units of enzyme per 200 glands, 30 min, 37°C.) **(5)** as in **(4)** but without enzyme. **(6)** *Xba*I digest of nuclei from L1-cells. (50 units of enzyme per A_{260} unit of nuclei, 30 min, 37°C.) **(7)** as in **(6)** but without enzyme **P** and λ are indicated in the legend to Figures 2 and 3.

BR 2 sequences, neither in the L1-cells, nor in the salivary glands. Similar observations were made by Spinelli *et al.* (1982) in histone gene chromatin, whereas Wu *et al.* (1979) and Levy and Noll (1981) found an increased rate of DNA degradation in active heat shock chromatin of *Drosophila* as compared with the degradation of DNA in bulk chromatin.

The BR 1 has a low activity in the oligopause and the pilocarpine-stimulated post-oligopause larvae used (Meyer *et al.*, 1983). The blot of the gel of Figure 2B was hybridized with a probe from the BR 1 (plasmid pCt21 or its genomic insert). The 246-bp repeat unit represented by the insert in pCt21 represents between 15% and 55% of the BR 1 gene and is tandemly arranged in one or a few blocks (Wieslander *et al.*, 1982). Sharp nucleosomal and polynucleosomal bands were seen for the DNA from nuclei of L1-cells (repeat size of ~175 bp, Figure 4, 1–4). The DNA from nuclei of salivary glands showed faint nucleosomal bands (repeat size of ~175 bp) superimposed on a smear (slots 5–8). Especially when slots 6 and 8 of Figures 3 and 4 are compared, a difference in the DNA pattern between BR 2 and BR 1 is obvious. In slot 8 of Figure 4 a distinct stop at the level of the trinucleosome and in slot 6 also a clear dinucleosomal band could be recognized, whereas in slots 6 and 8 of Figure 3 no stop or band was recognizable. The deproteinized DNA of BR 1 was degraded as a smear (Figure 4 slots 9–12).

Digestion of nuclei with XbaI

Ness *et al.* (1983) observed that among the restriction enzymes tested those with a 6-bp recognition sequence could digest only the coding region of the nucleolar chromatin of growing *Dictyostelium discoideum*, and not the nucleosome packed non-transcribed spacer. We tested 14 restriction enzymes with a 6-bp recognition sequence for their ability to cut *Chironomus* DNA within the BR 2 sequence, as tested by Southern hybridization with pCp31. Only *Xba*I gave suitable fragments. Figure 5 (slots 2–3) shows a partial *Xba*I digest of deproteinized DNA. Fragments of ~200 bp and multiples thereof were produced (for comparison Figure 5 slot 1 shows a micrococcal nuclease digest of L1-cell nuclei). These bands are explained by the single *Xba*I site in the tandem repeat of the BR 2 DNA sequence (Jäckle *et al.*, 1982; Sümegi *et al.*, 1982; Bäumlein *et al.*, 1982). The same bands as with free DNA (although probably with lower yield) were obtained when nuclei of salivary glands of pilocarpine-stimulated animals were digested with *Xba*I (Figure 5, slot 4), but nuclei incubated in parallel without restriction enzyme showed no corresponding DNA fragments (Figure 5, slot 5). On the other hand digestion of nuclei of L1-cells with *Xba*I did not lead to any detectable fragments hybridizable with pCp31 (Figure 5, slot 6, also with 75 units of enzyme added twice to the sample and a total digestion time of 60 min, data not shown) and also control nuclei incubated in parallel without enzyme did not show the corresponding bands (Figure 5, slot 7). Similar observations were described by Pfeiffer and Zachau (1980) studying the expressed kappa light chain of a myeloma cell line. (On the original negative of Figure 5 a very weak nucleosomal band pattern, probably due to the action of endogenous nuclease, is seen in slots 6 and 7.)

Discussion

Digestion of BR 2 chromatin in the salivary glands of pilocarpine-stimulated larvae by micrococcal nuclease did not produce discrete DNA fragments. Traces of nucleosomal bands were seen in chromatin from the less active glands of unstimulated larvae. The lack of periodicity, seen as a smear in electrophoresis, could be due either to undefined linker length between nucleosomes or to the absence of nucleosomes. The latter alternative could be due to a change in the nucleosomal structure without loss of the histones or to their total or partial absence. On our Southern blots the transfer

efficiency for fragments of 200 bp or less was low and (as in the case of Wu *et al.*, 1979) a distinction between the two cases is therefore not possible. However, Levy and Noll (1981) studying the chromatin of activated heat shock genes in *Drosophila* found not only a smear, but also a lack of a digestion stop at the mononucleosomal size which strongly suggests the absence of nucleosomes. This interpretation fits our electron microscopic observations which indicate that the template is almost entirely covered by RNA polymerases after pilocarpine treatment. [If RNA polymerase II were to protect from nuclease degradation a fragment similar in size to that protected by *Escherichia coli* RNA polymerase (42 bp, Schaller *et al.*, 1975) then we would not be able to detect it by our Southern blotting procedure.] We did not observe an increased rate of degradation of the transcribed *versus* the non-transcribed sequences, possibly because the transcribed sequences are tightly packed with RNA polymerases. The faint nucleosomal bands seen in the nuclease digests of non-pilocarpine-treated larvae could either be due to some elements of BR 2 chromatin with an unaltered nucleosomal organization or to a mixed population of both active and inactive BR 2 genes.

We also tested the accessibility of restriction enzymes to active and inactive BR 2 chromatin. Enzymes recognizing 4 bp cleaved the DNA in the BR 2 chromatin in both salivary glands and L1-cells (data not shown) and therefore cannot distinguish between inactive and active chromatin. We suggest that these enzymes are capable of cutting DNA wrapped around a nucleosome, while 6-bp enzymes are not, perhaps for topological reasons (Stasiak and Klopotowski, 1979). In agreement with Ness *et al.* (1983) we found that *Xba*I, which has a 6-bp recognition sequence, cleaved the BR 2 chromatin in salivary glands of pilocarpine-stimulated larvae and produced the same fragments as in deproteinized DNA. However, in the inactive chromatin of L1-cells, *Xba*I had no detectable action, again pointing to a fundamental difference in chromatin structure between inactive and transcribed BR 2 genes. This observation correlates with the electron microscopic findings that the chromatin axis between putative RNA polymerases appears smooth and thin, similar to the co-prepared free DNA. In contrast, Anderson *et al.* (1982), studying embedded and thin-sectioned specimens, proposed that the axis is covered, probably by histones. Whether at the site of transcription some (Baer and Rhodes, 1983) or most of the histones are absent (as suggested for nucleolar chromatin, Labhart and Koller, 1982; Labhart *et al.*, 1983), or whether histones still coat the DNA (Lohr, 1983), cannot be decided at present. A drastic disruption of the nucleosome structure might be necessary for DNA strand separation and transcription (Lilley *et al.*, 1979; Gould *et al.*, 1980), since nucleosomes and histones are known to inhibit RNA polymerase II activity (Karagyozov *et al.*, 1978; Weihe *et al.*, 1978; Lilley *et al.*, 1979; Wasylyk and Chambon, 1979).

Lamb and Daneholt (1979) found 16 RNP fibrils per μm of transcription unit (non-stimulated animals), which we could confirm in our experiments (data not shown) and which is significantly less than the 38 ($\pm$2) polymerases observed in the pilocarpine-treated larvae. We must point out that while the statistical analysis of Lamb and Daneholt (1979) was made at the level of the RNA fibers, our data from pilocarpine-treated larvae were obtained at the level of the RNA polymerases, because the thin RNP fibrils could not be counted unambiguously in these well-spread preparations (see Figure 1A and B). These electron microscopic observations and the faint nucleosomal repeat in the chromatin of non-stimulated larvae and the total absence of nucleosomal bands after pilocarpine treatment correlate with the stimulation of transcription by pilocarpine (see also Mähr *et al.*, 1980). The data suggest that proteins involved in transcription compete out histones organized into nucleosomes, in agreement with electron microscopic evidence (McKnight *et al.*, 1978; Scheer, 1978). Possible interactions between RNA polymerase and histones have been studied by Wasylyk and Chambon (1980) and Baer and Rhodes (1983). We propose that the 'active nucleosome' would be a nucleosome in close association and competition with the transcription machinery. The equilibrium between the binding of the transcription machinery on the one hand and the histone binding on the other hand could determine the transcription rate. Scheer *et al.* (1979) and Einck and Bustin (1983) have shown that antibodies against histones inhibit transcription of RNA polymerase II. This inhibition could be explained by a shift in such an equilibrium, i.e., by a stabilization of the nucleosomes by the antibody binding.

Materials and methods

Materials

C. tentans larvae (strain $2L_K$) were from our inbred laboratory culture (Meyer *et al.*, 1983, for culturing conditions). The epithelial *C. tentans* cell line (L1) was established from embryos by Wyss (1982).

Plasmid pCp31 contains a repetitive sequence from the BR 2 of *C. pallidivittatus* (Jäckle *et al.*, 1982). Plasmid pCt21 contains a sequence from the BR 1 from *C. tentans* (Wieslander *et al.*, 1982). PM-2 DNA was purified according to Espejo *et al.* (1969). Micrococcal nuclease was from Worthington, reverse transcriptase, proteinase K and restriction enzyme *Xba*I from Boehringer, and oligo(dT)-cellulose from P.L.Biochemicals. The kit for nick translation was from Amersham. Biotinylated dUTP and goat anti-biotin IgG were from Enzo Biochem. Inc. (NY). Rhodamin-labelled rabbit anti-goat IgG was from Cappel Lab. (Cochranville, PA). Gene Screen was purchased from NEN (New England Nuclear).

Buffer A1 (modified from Hewish and Burgoyne, 1973) contained 15 mM Tris-HCl at pH 7.0, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA and 0.5 mM dithiothreitol (DTT). Buffer A4 contained 15 mM Tris-HCl at pH 7.4, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine and 0.1 mM EDTA. Buffer A-Mn contained 15 mM Tris-HCl at pH 7.6, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM $MnCl_2$. Glancy's buffer is 90 mM KCl, 60 mM NaCl, 10 mM triethanolamine chloride at pH 7.6.

Preparation of nuclei

Post-oligopausing *C. tentans* larvae were stimulated with pilocarpine as described by Meyer *et al.* (1983). In some experiments, unstimulated larvae from oligopause were used (Meyer *et al.*, 1983). The subsequent steps were performed on ice. Salivary glands ($\sim$450 in each experiment) were isolated by hand in buffer A1 containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF). In the experiments with the cell line L1 $\sim 1-2 \times 10^7$ cells were pelleted at 3000 *g* for 3 min and then washed once in buffer A1. Glands and L1-cells were lysed in buffer A1 containing 0.5% Triton X-100 by pipetting with a siliconised tip for 5–10 min. Nuclei were pelleted at 500 *g* (salivary glands) or 10 000 *g* (cell cultures), washed one or two times in buffer A4 containing 0.1% Triton X-100, and once in buffer A4. The amount of nuclei obtained from cell culture was estimated by measuring the absorbance at 260 nm (A_{260}) of a small aliquot in 1 N NaOH.

Digestion of nuclei with micrococcal nuclease

To nuclei in buffer A4 (salivary glands: nuclei of $\sim$450 glands/ml; cell culture: $A_{260} \sim 10$) $CaCl_2$ was added to 1 mM. The sample was incubated at 37°C for 2 min, micrococcal nuclease was then added. The reaction was stopped with EDTA and SDS brought to a concentration of 5 mM and 0.5%, respectively.

Digestion of nuclei with restriction enzyme XbaI

Nuclei washed in buffer A4 were resuspended in 50 μl of 150 mM NaCl, 6 mM Tris-HCl at pH 7.9, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol. Diges-

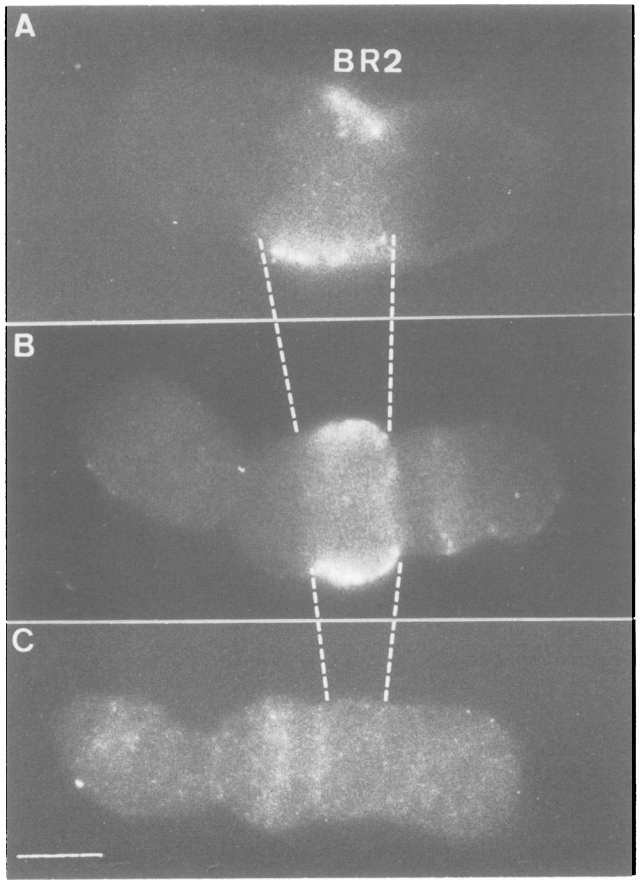

Fig. 6. *In situ* hybridization of various biotin labelled DNA probes to the 4th chromosome of *C. tentans* salivary glands. **(A)** Hybridization with pCp31, using a hybridization temperature of 68°C. **(B)** Hybridization with a cDNA prepared from poly(A)-containing RNA from salivary glands from pilocarpine-stimulated animals (hybridization temperature 58°C). **(C)** Hybridization with a cDNA prepared from poly(A)-containing RNA from L1-cell (hybridization temperature 58°C). The biotin-labelled probes were visualized with a primary anti-biotin antibody and rhodamin-labelled secondary antibody. Bar represents 10 μm.

tion was at 37°C for 30 min. The reaction was stopped with 300 μl of 0.5% SDS, 50 mM Tris-HCl at pH 7.5, 5 mM EDTA, 100 mM NaCl.

DNA extraction and analyses

Proteinase K was added to a concentration of 150 μg/ml to the stopped nuclease reaction, and the sample was digested at 50°C for 2 h. After two phenol and one chloroform extraction, the sample was treated with RNase A (100 μg/ml) at 37°C for 20 min. It was then extracted with phenol and chloroform once each and was ethanol precipitated.

The DNA was electrophoresed in vertical 3 mm thick agarose gels (1.5%) in 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA. After staining with ethidium bromide (0.5 μg/ml) the gels were transferred as described by Southern (1975) onto Gene Screen or as described by Sun *et al.* (1982) onto DBM-paper prepared according to Levy *et al.* (1980). The filters were prehybridized at 68°C for 4 – 6 h in 15 – 30 ml of 6 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5 x Denhardt's solution (Denhardt, 1966), 0.5% SDS and 100 μg/ml of salmon sperm DNA. Hybridization was for 16 h at 68°C in the above solution containing ~0.5 μg of [^{32}P]nick translated plasmid DNA (~5 x 10^7 c.p.m./μg) or ~30 ng of the excised insert (~3 x 10^8 c.p.m./μg) (Tabak and Flavell, 1978). The filters were washed at 68°C for 5 min and then for 60 min in 0.3 x SSC, 0.2% SDS, for 60 min in 0.5 M sodium phosphate at pH 7.0 and then twice for 60 min in 0.3 x SSC, 0.2% SDS. Autoradiographic exposure was on activated Fuji RX with an Ilfo Rapid Front Screen at −80°C. The filters were used for several hybridizations. Used filters were soaked in 0.3 x SSC, 0.2% SDS, were then treated with 20 ml formamide at 60°C for 90 min and were washed in 2 x SSC.

Poly(A)$^+$ RNA extraction

Total RNA from salivary glands was isolated as described by Weber *et al.* (1983). By the same procedure, total nucleic acids from L1-cells were extracted. Poly(A)$^+$ RNA was then separated over an oligo(dT)-column (Maniatis *et al.*, 1982).

Electron microscopy

For the preparation of one grid, two salivary glands were isolated by hand in Glancy's buffer. The isolated glands were incubated at room temperature for 3 min in 50 μl of Glancy's buffer containing 1% Triton X-100. They were then transferred into an equal volume of buffer A-Mn and kept for another 3 min. The glands were squashed in 15 μl buffer A (Hewish and Burgoyne, 1973) and glycerol (v/v 1:9) with a siliconized coverslip. After removing the coverslip about five chromosomes (No. IV) were picked up with a micropipette mounted in a micromanipulator (Leitz) and transferred into 20 μl containing 1 mM triethanolamine chloride (pH 9), 0.2 mM EDTA. After 30 min at room temperature the whole droplet was transferred with a siliconized tip into the microcentrifuge tube as described by Labhart and Koller (1981).

In some experiments BR 2 was cut out as described by Frey *et al.* (1982). The cutting was performed in buffer A (Hewish and Burgoyne, 1973) and glycerol (v/v = 1:9).

In situ hybridizations

We used *in situ* hybridization to test the specificity of hybridization of pCp31 in *C. tentans* chromosomes. Plasmid DNA was biotin labeled by nick translation with biotinylated dUTP according to the suppliers recommendations and cDNA was labeled by reverse transcribing poly(A)$^+$ RNA (Katcoff *et al.*, 1980), but in the presence of biotinylated dUTP according to the suppliers recommendations. *In situ* hybridization was carried out according to method 2 of Langer-Safer *et al.* (1982) at a temperature of 68°C or 58°C. The secondary antibody was rhodamin labelled. The samples were observed by epifluorescent illumination with a 63 x Neofluar (Zeiss) objective lens.

As shown in Figure 6A under the hybridization conditions used (68°C) the biotin-labelled probe hybridized exclusively in the BR 2, and only when less stringent hybridization conditions were used (58°C) was a faint labelling of BR 1 also observed (data not shown). The expression of the BR 2 gene was also examined by *in situ* hybridization. A biotin-labelled cDNA prepared from poly(A)$^+$ RNA extracted from salivary glands of pilocarpin-stimulated animals hybridized strongly in the BR 2 (Figure 6B) suggesting a high abundance of BR 2 transcripts in salivary glands. On the other hand a cDNA prepared from poly(A)$^+$ RNA from L1-cells showed no labelling of the BR 2 above background (Figure 6C) indicating the absence of BR 2 transcripts, probably due to inactivity of BR 2 in these cells. These L1-cells were therefore used as reference for the inactive BR 2 chromatin.

Acknowledgements

We thank B.Daneholt for the generous gift of plasmids pCt63 (Sümegi *et al.*, 1982) and pCt21 (Wieslander *et al.*, 1982) H.Mayer-Rosa and F.Gatzka for excellent technical help and P.J.Ness for critical reading of the manuscript. This work was supported by Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung, grants to M.Lezzi and Th.Koller.

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Received on 5 March 1984; revised on 6 April 1984