Characterization of poly(ADP-ribosyl)ated domains of rat pachytene chromatin

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Poly(ADP-ribosyl)ation of nuclear proteins was several-fold higher in the pachytene spermatocytes than in the premeiotic germ cells of the rat. Among the histones of the pachytene nucleus, histone subtypes H2A, H1 and H3 were poly(ADP-ribosyl)ated. Based on the immunoaffinity fractionation procedure of Malik, Miwa, Sugimara & Smulson [(1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2554–2558] we have fractionated DNAase-II-solubilized chromatin into poly(ADP-ribosyl)ated chromatin (PAC) and non-poly(ADP-ribosyl)ated chromatin (non-PAC) domains on an anti-[poly(ADP-ribose)] IgG affinity matrix. Approx. 2.5% of the pachytene chromatin represented the PAC domains. A significant amount of [α - 32 P]dATP-labelled pachytene chromatin (labelled *in vitro*) was bound to the affinity matrix. The DNA of pachytene PAC domains had internal strand breaks, significant length of gaps and ligatable ends, namely 5'-phosphoryl and 3'-hydroxyl termini. On the other hand, the PAC domains from 18 h regenerating liver had very few gaps, if any. The presence of gaps in the pachytene PAC DNA was also evident from thermal denaturation studies. Although many of the polypeptides were common to the PAC domains of both pachytene and regenerating liver, the DNA sequences associated with these domains were quite different. A 20 kDa protein and the testis-specific histone H1t were selectively enriched in the pachytene PAC domains. The pachytene PAC domains also contained approx. 10% of the messenger coding sequences present in the DNAase-II-solubilized chromatin. The pachytene PAC domains, therefore, may represent highly enriched DNA-repair domains of the pachytene nucleus.

INTRODUCTION

Poly(ADP-ribose), the third polynucleotide other than DNA and RNA, present only in eukaryotic cells, is covalently linked post-translationally to nuclear proteins by the chromatin-bound enzyme poly(ADP-ribose) polymerase (EC 2.4.2.30) (Gaal & Pearson, 1985). Despite the extensive studies on the possible functions of this modification process, it has not been possible to make definitive conclusions regarding its biological significance. However, two well-established facts associated with this phenomenon are (1) the rate and extent of this modification process is closely linked to DNA-repair activity (Gaal & Pearson, 1985) and (2) poly(ADP-ribosyl)ation of nucleosomal histone H1 relaxes the chromatin structure in a reversible manner (deMurcia *et al.*, 1986). Further, several nuclear proteins including many enzymes are shown to be regulated by this modification process (Gaal & Pearson, 1985; Yoshihara *et al.*, 1985).

Pachytene spermatocytes represent an important stage of meiotic prophase of the germ cells wherein genetic recombination takes place between the paired parental homologous chromosomes. Most of the premeiotic DNA synthesis is completed in preleptotene spermatocytes, while only a small fraction of the genome replicates at the zygotene stage (Stern & Hotta, 1980). The only DNA synthetic activity associated with the pachytene

spermatocytes is DNA repair linked to the recombination process (Stubbs & Stern, 1986). Based on the presumption that such recombination repair domains may be poly(ADP-ribosyl)ated preferentially over the rest of chromatin, we have sought to isolate and characterize the poly(ADP-ribosyl)ated domains of pachytene chromatin. For this purpose, we have employed the immunoaffinity technique developed by Malik *et al.* (1983). The results presented here show that poly(ADP-ribosyl)ation of nuclear proteins is several-fold higher in pachytene nuclei than in premeiotic spermatogonial nuclei. Further, several molecular properties of affinity-purified poly(ADP-ribosyl)ated domains of pachytene chromatin suggest that they may represent the recombination-linked DNA-repair domains.

MATERIALS AND METHODS

Materials

Male albino rats of this Institute strain (body weight 150 ± 10 g) were used in all the studies. DNAase II, micrococcal nuclease, pancreatic RNAase A, proteinase K, NAD, phenylmethanesulphonyl fluoride and collagenase (Type IV) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Calf intestinal phosphatase was from Boehringer-Mannheim, while *E. coli* DNA polymerase I, Klenow polymerase and polynucleotide kinase were from New England Biolabs. [γ - 32 P]dATP (specific activity

Abbreviations used: PAC, poly(ADP-ribosyl)ated chromatin; DTT, dithiothreitol; TCA, trichloroacetic acid; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline.

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3000 Ci/mmol), [α - 32 P]ATP (specific activity 3000 Ci/mmol) and [14 C]NAD (nicotinamide [14 C]adenine dinucleotide, specific activity 290 mCi/mmol) were from Amersham International, Amersham, Bucks., U.K. Nitrocellulose sheets (BA87) were from Schleissner & Schuell.

Isolation of total nuclei and purification of pachytene and spermatogonial nuclei

Total nuclei from liver, regenerating liver 18 h after partial hepatectomy and testes of rats were isolated essentially following the procedure described by Rao *et al.* (1982). Pachytene-stage nuclei from adult rat testes and spermatogonial nuclei from testes of 10-day-old rats were purified by employing the STAPUT technique as described by Rao *et al.* (1983).

Incorporation of [14 C]NAD into nuclear proteins

The rates of poly(ADP-ribosylation) of nuclear proteins were measured in different nuclear preparations according to the method of Sugimura *et al.* (1971). Approx. 10 absorption (A_{260}) units each of purified pachytene and spermatogonial nuclei were taken in a final volume of 0.5 ml of buffer A [100 mM-Tris/HCl, pH 8.0/30 mM-MgCl₂/1 mM-dithiothreitol (DTT)] containing 0.2 mM cold NAD and 1 μ Ci of [14 C]NAD in a siliconized Corex tube, and the reaction was carried out at 25 °C with agitation. At various time points, 5 μ l aliquots were removed, in duplicate, and were spotted on to Whatman No. 3 filter paper circles wetted with 50% trichloroacetic acid (TCA). The filters were air dried and successively washed with cold 10% TCA, ethanol and ether. After air drying, the radioactivity in the filters was counted in an LKB Rack Beta Counter using 0.5% PPO in toluene as the scintillator. The TCA-precipitable radioactivity represents mostly poly(ADP-ribosylated) proteins, since this is the only reaction catalysed in the eukaryotic cell nucleus using NAD as the substrate (Smulson & Sugimura, 1984).

Purification of poly(ADP-ribose) polymer and raising antibodies in rabbit

Poly(ADP-ribose) was synthesized enzymically *in vitro* as described earlier (Sugimura *et al.*, 1971), with testicular nuclei as the source of poly(ADP-ribose) polymerase. Purification of the polymer from the nuclei after incubation with NAD was carried out by treating with Pronase, pancreatic DNAase I, RNAase A and spleen phosphodiesterase as described by Shima *et al.* (1970) and Sugimura *et al.* (1971) and subsequently chromatographed on a hydroxyapatite (Bio-Rad) column. The purity of the polymer was checked according to the method of Shima *et al.* (1970), and the average chain length was found to be approx. 25 residues.

Poly(ADP-ribose) was coupled to methylated bovine serum albumin and antibodies were produced in rabbit as described by Kanai *et al.* (1974). The presence of specific antibodies in the antiserum and their specificity were checked by the Ouchterlony double immunodiffusion technique (Ouchterlony, 1958). The IgG fraction from the antiserum was purified by ammonium sulphate precipitation and negative absorption on DEAE cellulose (Livingston, 1974).

Indirect immunofluorescence assay

Immunofluorescence of stained nuclei was carried out as described by Markose & Rao (1985), except that the nuclei were incubated with 1 mM-NAD at 25 °C for 10 min in TKM buffer (50 mM-Tris/HCl, pH 7.5/25 mM-KCl/5 mM-MgCl₂) containing 1 mM-DTT prior to incubation with anti-[poly(ADP-ribose)] IgG.

Digestion of nuclei with DNAase II and immunofractionation of DNAase-II-released chromatin on an anti-[poly(ADP-ribose)]IgG column

Approx. 30 A_{260} units of pachytene nuclei or regenerating liver nuclei were first incubated with 1 mM-NAD plus buffer A for 10 min at 25 °C. The nuclear pellet obtained after centrifugation was washed once with DNAase II digestion buffer [20 mM-sodium acetate, pH 6.0/0.25 mM-Na₂EDTA/0.1 mM-phenylmethylsulphonyl fluoride (PMSF)] and then suspended in the same buffer at a nuclei concentration of 25–30 A_{260} units/ml. Digestion was carried out by using 4 units of DNAase II/ A_{260} unit of nuclei at 25 °C for various time periods to get the required percentage of digestion, and stopped by increasing the pH to above 7.5 with 1 M-Tris/HCl buffer, pH 8.8, as described earlier (Hotta & Stern, 1981). The supernatant obtained after centrifugation at 8000 g represented the DNAase-II-solubilized chromatin.

For selective labelling of the poly(ADP-ribosylated) chromatin domains, nuclei were incubated with 2–3 μ Ci of [14 C]NAD/30–40 A_{260} units of nuclei for 10 min at 25 °C prior to the DNAase II digestion. For labelling of the repair domains in pachytene nuclei and the replicating domains in regenerating liver nuclei, the appropriate pure nuclear preparations were incubated in a final volume of 1.5 ml containing 30 A_{260} units of nuclei/50 mM-Tris/HCl, pH 8.0/5 mM-MgCl₂/2.5 mM-DTT/0.3 M-nuclease-free sucrose/10 mM each of dGTP and TTP/50 μ Ci of [α - 32 P]dATP for various time periods at 34 °C prior to DNAase II digestion.

Anti-[poly(ADP-ribose)] IgG was coupled to CNBr-activated Sepharose 4B as described by Malik *et al.* (1983). The affinity gel matrix was suspended in phosphate-buffered saline (PBS; 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) containing 0.1 mM-PMSF and stored at 4 °C with 0.001% sodium azide as the preservative.

The DNAase-II-solubilized chromatin was either dialysed against PBS or made to final 1 $\times$ PBS concentration with 10 $\times$ PBS and applied to the affinity gel matrix at 4 °C. The column was washed with PBS at a flow rate of 2 ml/h. The eluate was monitored at 260 nm until there was no more absorbance. The matrix was washed with PBS containing 1.6 M-KSCN. Immediately after elution, the fractions containing the bound chromatin were pooled and extensively dialysed against PBS to remove KSCN. The absence of KSCN was ascertained by colour reaction with FeCl₃.

Electrophoretic analysis of DNA and proteins of various chromatin fractions

The DNA of the various chromatin fractions was obtained after treatment with proteinase K and DNAase-free RNAase and subsequent phenol/chloroform (1:1, v/v) extraction and ethanol precipitation (Rill *et al.*, 1978). DNA samples were analysed on a 6% non-

denaturing polyacrylamide gel (Maniatis *et al.*, 1975). For analysis of the proteins, various chromatin fractions were digested in Tris buffer (50 mM-Tris/HCl, pH 7.5/5 mM-MgCl₂, 0.1 mM-PMSF with pancreatic DNAase I (10 µg of enzyme/4 A₂₆₀ units of chromatin) for 40 min at 37 °C, and the proteins were precipitated with 2.5 vol. of ethanol at -20 °C. The pellets obtained after centrifugation were washed once with 80 % ethanol, dissolved in SDS sample buffer and analysed on an SDS/polyacrylamide gel (5–15 % gradient) (Laemmli, 1970). Acid-soluble nuclear proteins labelled with [¹⁴C]NAD were analysed on a Triton X-100-acid-urea polyacrylamide gel (Alfageme *et al.*, 1974), after which the gel was subjected to fluorography.

Hybridization of various chromatin DNA fractions with nick-translated pachytene poly(ADP-ribosyl)ated chromatin DNA as the probe

Different concentrations of DNA samples of the poly-(ADP-ribosyl)ated chromatin (PAC) and non-PAC DNA of pachytene and regenerating liver as well as the DNA associated with the DNAase-II-undigested chromatin were denatured and hybridized with nick-translated (Maniatis *et al.*, 1982) pachytene PAC DNA (specific activity of the probe was 1×10^7 c.p.m./µg of DNA) by using the DNA dot-blot technique (Meinkoth & Wahl, 1984).

5'-End labelling, DNA gap filling and detection of internal strand breaks

DNAase II generates 5'-OH recessive ends. Therefore, for labelling the 5'-ends on DNAase-II-digested chromatin DNA, the procedure described for blunt-end labelling (Maniatis *et al.*, 1982) was used. DNA from PAC and non-PAC fractions from pachytene nuclei were treated with calf intestinal phosphatase which was later inactivated. Both the phosphorylated and unphosphorylated DNA were end-labelled with 50 µCi of [³²P]ATP and 10 units of polynucleotide kinase for 1 h at 37 °C, after which the DNA was extracted with phenol/chloroform (1:1, v/v) and the free label was removed by Sephadex G-50 chromatography.

For carrying out the gap-filling reaction, 0.5 µg each of pachytene PAC and non-PAC DNA were incubated in the nick-translation buffer containing 10 µg each of dGTP, dCTP and TTP and 25 µCi of [³²P]dATP along with 1 unit of Klenow fragment of *E. coli* DNA polymerase I for 30 min at room temperature. After incubation, the reaction was stopped with Na₂EDTA, extracted with phenol/chloroform (1:1, v/v) and the DNA was separated from the free label as mentioned above.

The presence of internal strand breaks in the DNA of pachytene PAC and non-PAC fractions was analysed by subjecting the 5'-end-labelled fragments to electrophoresis on a 6 % polyacrylamide gel in the first dimension and subsequently in the second dimension under denaturing conditions (Maniatis *et al.* 1975). Briefly, the gel lanes from the first-dimension gel were carefully sliced and incubated with 100 % deionized formamide. After two or three changes of formamide, the gel piece was aligned on a 5 % polyacrylamide gel containing 98 % formamide and 0.02 M-sodium phosphate, pH 7.5, which had been pre-electrophoresed for 5 h. The electrophoresis buffer (0.02 M-sodium phosphate, pH 7.5) was recycled during the run. The electrophoresis was

carried out at a constant voltage of 10 V/cm using Bromophenol Blue as the tracking dye. After the electrophoretic run, the gel was removed, covered with Saran Wrap and subjected to autoradiography at 4 °C.

Thermal denaturation of DNA from various chromatin fractions

For this purpose, various chromatin fractions were digested with pancreatic RNAase A and proteinase K, and the DNA extracted, as described above, was analysed by thermal denaturation in a Beckman DU 8B spectrophotometer. The temperature range was from 30–90 °C. The rate of increase in the temperature range was 0.5 °C/min, while the absorbance at 260 nm was recorded at 0.5 or 1 °C intervals. DNA melting was carried out in 0.1 × SSC (15 mM-NaCl/1.5 mM-Na₃ citrate, pH 7.0). The DNA samples were in the concentration range 0.3–0.6 A₂₆₀ units/ml.

Isolation of poly(A⁺) RNA from pachytene cells and synthesis of radioactive cDNA probe

Pachytene cells were purified using the procedure of Gold *et al.* (1983) with minor modifications. Briefly, the testicular tissue was suspended in Krebs–Ringer bicarbonate buffer, passed through a 20 ml syringe without the needle and centrifuged. The process was then repeated. The pellet was suspended in Krebs–Ringer buffer (1 g of tissue/10 ml) and then treated with collagenase (200 µg/ml) at 37 °C for 20 min. The suspension was filtered through two layers of cheesecloth and the filtrate containing single cell suspension was subjected to STAPUT sedimentation at 4 °C using Krebs–Ringer buffer containing 1–3 % bovine serum albumin as the gradient. After 3 h, the pachytene cells were siphoned out and their purity was checked under a light microscope. Poly(A⁺) RNA was isolated from these pachytene cells and cDNA synthesis was carried out as described by Maniatis *et al.* (1982) with minor modifications. Purified poly(A⁺) RNA (10 µg) was taken in 100 µl of 100 mM-Tris/HCl, pH 8.3/10 mM-MgCl₂/140 mM-KCl/10 µg of oligo(dT)/20 mM-β-mercaptoethanol/1 mM-vanadyl ribonucleoside complex/1 mM each of dGTP, dCTP, dTTP/0.02 M unlabelled dATP containing 100–150 µCi of [³²P]dATP/50 units of reverse transcriptase. The incubation was done for 3 h at 42 °C, after which the reaction was stopped by adding 3 µl of 0.5 M-Na₂EDTA, pH 7.0, followed by 1.25 µl of 3 M-NaOH. After incubating for 1 h at 65 °C, the mixture was neutralized by adding 25 µl each of 1 M-Tris/HCl, pH 8.0, 1 M-HCl and subjected to phenol/chloroform (1:1, v/v) extraction. The free nucleotides were removed by Sephadex G-50 chromatography and the cDNA was stored with 5–10 µg of cold sperm DNA at -20 °C.

Transcription *in vitro* and analysis of transcriptional activity of the fractionated chromatin

Nuclear transcription was carried out *in vitro* as described by Guertin *et al.* (1983) with minor modifications. All the operations were carried out under RNAase-free conditions. All the buffers, including nuclei isolation buffers, contained 1 mM-NAD. Purified pachytene nuclei were incubated in poly(ADP-ribosylation) buffer with NAD for 10 min as described above, and washed once with Hepes buffer, pH 7.5, containing 5 mM-magnesium acetate and 40 % glycerol. After centrifugation at 2500 g for 10 min the nuclear pellet was

suspended in the same buffer (0.6 ml/20 A_{260} units) and kept at -70°C for 10–12 h. Later, this suspension was thawed and incubated in a final volume of 1.5 ml containing 20 mM-Hepes, pH 7.5/5 mM-magnesium acetate/150 mM-NAD/1 mM- MnCl_2 /2 mM-DTT/0.1 mM- Na_2EDTA /0.4 mM each of ATP, GTP and CTP/2 mM-creatine phosphate/4.5 units of creatine phosphokinase/16% glycerol/500–700 units of Rnasin/100 μCi of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$. The reaction was carried out at 25°C for 30 min. Later, nuclei were pelleted at 2500 g for 10 min and digested with DNAse II to obtain 8–10% digestion as described above. Total chromatin digest obtained after centrifugation was loaded on a Sephadex G-50 column and the chromatin peak fractions in the void volume were pooled and fractionated on the anti-[poly(ADP-ribose)] IgG affinity matrix as described above. Suitable aliquots of the bound and unbound fractions were taken into methoxyethanol/toluene (1:1 v/v) scintillator and the radioactivity present was counted.

RESULTS

Poly(ADP-ribosyl)ation of pachytene and spermatogonial nuclear proteins

Pachytene and spermatogonial nuclei were isolated from adult and 10-day-old rat testes respectively by employing the STAPUT technique. The preparations were greater than 90% pure as judged by phase-contrast microscopy (Rao *et al.*, 1983). The kinetics of incorporation of $[\text{}^{14}\text{C}]\text{NAD}$ into proteins of pachytene and spermatogonial nuclei (Fig. 1) indicate that both the rate and extent of incorporation into pachytene nuclear proteins was greater than that into spermatogonial proteins. After 60 min of incubation, the total incorporation was approx. 8-fold higher in pachytene nuclei than in spermatogonial nuclei.

Theoretically it is possible that the TCA-precipitable radioactivity might also include long poly(ADP-ribose) polymers containing extended $[\text{}^{14}\text{C}]\text{ADP-ribose}$ free of proteins. However, this is very unlikely as poly(ADP-ribose) polymerase can catalyse incorporation of the

ADP-ribose moiety only in acceptor proteins or to extend chains which are already linked to proteins. Even the protein-free poly(ADP-ribose) extended with $[\text{}^{14}\text{C}]\text{ADP-ribose}$ from $[\text{}^{14}\text{C}]\text{NAD}$, if at all present, truly reflects the poly(ADP-ribose) polymerase activity. Considering the fact that the pachytene nucleus has double the DNA content of the spermatogonial nucleus, it can be inferred that the net incorporation per pachytene nucleus is nearly 16-fold higher than for each spermatogonial nucleus. This conclusion is further supported by the results obtained with indirect immunofluorescence. For this purpose, highly specific antibodies were raised

Fig. 1. Kinetics of incorporation of $[\text{}^{14}\text{C}]\text{NAD}$ into nuclear proteins

Approx. 10 A_{260} units of spermatogonial (●) or pachytene (○) nuclei were incubated with 1 μCi of $[\text{}^{14}\text{C}]\text{NAD}$ and 0.2 mM unlabelled NAD at 25°C . Aliquots were withdrawn at different time intervals and TCA-precipitable radioactivity was determined.

Fig. 2. Ouchterlony double immunodiffusion pattern with anti-[poly(ADP-ribose)]IgG

The central wells in (a) and (b) contained 500 μg of anti-[poly(ADP-ribose)] IgG. (a) Wells 1, 2 and 3 contained 10, 20 and 30 μg respectively of pure poly(ADP-ribose) polymer; well 4, methylated bovine serum albumin (10 μg); well 5, calf thymus DNA (10 μg); well 6, rat testis cytoplasmic RNA (10 μg); well 7, poly(A) (10 μg); well 8, NAD (10 μg). The precipitin line could be seen within 8 h, after which the gel was stained with Coomassie Brilliant Blue and destained. (b) Well 1, methylated DNA (15 μg); well 2, single-stranded M13 phage DNA (15 μg); wells 3 and 4, 15 and 30 μg respectively of riboflavin carrier protein (containing 18% carbohydrate).

Fig. 3. Indirect immunofluorescence pattern of testicular nuclei with anti-[poly(ADP-ribose)]IgG

Total testicular nuclei were treated with anti-[poly(ADP-ribose)] IgG (2 mg) and then subsequently with FITC-labelled goat anti-rabbit IgG (200 μ g, NL-Cappel Laboratories Inc., Cochranville, PA, U.S.A.) and then examined in a Zeiss Photomicroscope III and photographed with ORWO NP 27 film (ASA 400). (a), (b) and (c) represent the phase-contrast micrographs, while (d), (e) and (f) represent the fluorescence pattern. P, pachytene; Sg, spermatogonia; RS, round spermatide; TS, testicular sperm. $\times 240$.

against poly(ADP-ribose) in rabbits and the specificity of antibody recognition is illustrated in Fig. 2. As judged by the Ouchterlony immunodiffusion test, it is clear that the anti-[poly(ADP-ribose)] antibody was highly specific to poly(ADP-ribose). Competition experiments employing the nitrocellulose filter binding assay using [14 C]NAD-labelled poly(ADP-ribose) also confirmed the specificity of the anti-[poly(ADP-ribose)] antibodies. We would like to mention here that the antibodies were raised against highly purified poly(ADP-ribose) prepared according to Shima *et al.* (1970), a procedure including extensive treatment with Pronase. Hence it is very unlikely that antibodies are directed towards any nuclear proteins, including histones. Moreover, histones did not significantly compete (about 5%) with the binding of [14 C]poly(ADP-ribose) to antibodies in the nitrocellulose filter binding assays. We have not been able to do more

sensitive e.l.i.s.a. assays for poly(ADP-ribose) to carry out cross-reactivity studies.

From the results of immunofluorescence experiments, shown in Fig. 3, it is evident that maximum fluorescence was observed with pachytene nuclei. While the premeiotic spermatogonial nuclei showed much reduced fluorescence, the postmeiotic, round spermatid nuclei as well as the testicular sperm did not show any fluorescence. From these results we believe that anti-[poly(ADP-ribose)] antibodies are not cross-reacting with histones. If they were cross-reacting, then one would not have observed differential fluorescence between spermatogonia, pachytene and round spermatid nuclei. In a similar study, Coromines & Mezquita (1986) have shown that a mixture of premeiotic and meiotic germ cell nuclei of a rooster had high levels of poly(ADP-ribose) polymerase activity when compared with postmeiotic germ cell nuclei.

Fig. 4. Polyacrylamide electrophoretic analysis of poly(ADP-ribosyl)ated histones

Pachytene (P) or spermatogonial (Sg) nuclei (20 A_{260} units) were incubated with 5 μ Ci of [14 C]NAD as described in the Materials and methods section for 20 min without any unlabelled NAD. Histones were extracted with 0.25 M-HCl and the acid-soluble proteins (75 μ g) were subjected to electrophoresis on a Triton-acid-urea 12% polyacrylamide gel. (a) Amido Black stained pattern and (b) fluorogram of the same gel.

Analysis of proteins that were poly(ADP-ribosyl)ated following incubation with [14 C]NAD in the pachytene nucleus could not be carried out on a SDS/polyacrylamide gel because of degradation of the polymer under the conditions of electrophoresis which resulted in a smear of radioactivity in the fluorographic pattern. However, acid-soluble [14 C]NAD-labelled proteins were analysed on a Triton X-100-acid-urea gel to get an idea of the histones that were poly(ADP-ribosyl)ated in the pachytene nucleus. A fluorographic pattern of such a gel is shown in Fig. 4 and it can be seen that histones H1, TH1 (H1a + H1t), H2A subtypes and H3 were (ADP-ribosyl)ated while histones H2B, TH2B and H4 were not labelled during incubation with [14 C]NAD.

Immunofractionation of soluble chromatin from pachytene and regenerating liver into PAC and non-PAC domains

Malik *et al.* (1983) have fractionated HeLa cell chromatin on an anti-[poly(ADP-ribose)] IgG column to yield PAC and non-PAC domains, and have further shown that the column-bound PAC domains had internal strand breaks in the DNA, suggesting that they contained the ongoing repair domains. Hence, to characterize the PAC domains of the pachytene nucleus, we have fractionated the soluble chromatin on an anti-[poly(ADP-ribose)] IgG affinity matrix.

Soluble chromatin was prepared from [14 C]NAD

labelled pachytene nuclei by digesting them with DNAase II to obtain 15% digestion. DNAase II was preferred over micrococcal nuclease mainly because it lacks the exonuclease activity and hence no chromatin DNA is lost during preparation of soluble chromatin. The fractionation profile of DNAase-II-solubilized chromatin on an anti-[poly(ADP-ribose)] IgG affinity matrix is shown in Fig. 5. More than 90% of the total input radioactivity was bound to the affinity matrix, from which it could be eluted with 1.6 M-KSCN, which itself could be removed by extensive dialysis against PBS for further analysis of the bound chromatin fraction. When pachytene nuclei were digested to different extents with DNAase II and then fractionated on the affinity column, approx. 17% and 14% of the DNAase-II-solubilized chromatin was bound to the affinity matrix after 13 and 37% digestion respectively. Therefore, it seems that poly(ADP-ribosyl)ated chromatin domains are more accessible to DNAase II and are preferentially released at early time points of digestion. Malik *et al.* (1983) have reached similar conclusions in their studies. Taking into account the percentage digestion of pachytene chromatin, it was estimated that approx. $2.5 \pm 0.3\%$ (four independent determinations) of pachytene chromatin represented the PAC domains.

Poly(ADP-ribosyl)ation of nuclear proteins has also been implicated in DNA replication (Jump *et al.*, 1979). With a view to comparing the PAC domains of pachytene with those of actively replicating chromatin, we have carried out parallel studies with nuclei from 18 h regenerating liver under identical conditions to those described above for pachytene nuclei. The amount of regenerating liver chromatin bound to the affinity matrix was estimated to be approx. 5–7% of the total regenerating liver chromatin. The polyacrylamide-gel electrophoretic pattern of DNA associated with PAC and non-PAC domains of pachytene and regenerating rat liver nuclei is shown in Fig. 5(b).

In order to see if the pachytene PAC domains contain the DNA-repair domains, isolated pachytene nuclei were incubated with [α - 32 P]dATP prior to DNAase II digestion. The soluble chromatin obtained at 15% digestion was passed through a column of Sephadex G-50 to remove the free label and then subjected to immunofractionation. A significant amount of radioactivity was bound to the column which could be eluted with 1.6 M-KSCN (Fig. 5). However, a substantial amount of radioactivity was present in the non-PAC fraction which still seems to retain some non-specifically absorbed [α - 32 P]dATP. It has been shown earlier by Stubbs & Stern (1986) that [3 H]thymidine labelling of mouse pachytene DNA is insensitive to treatment with hydroxyurea and hence represents repair synthesis. We have recently confirmed these observations in the rat pachytene spermatocytes as well, and therefore we believe that labelling of pachytene DNA *in vitro* with [α - 32 P]dATP also represents repair synthesis. Determination of the specific activity of DNA after extraction with phenol/chloroform and several precipitations with ethanol showed that the non-PAC fraction had approx. 2–3-fold higher specific activity than the PAC fraction (Table 1). The exact significance of this data is difficult to ascertain at present.

When similar studies were done with nuclei from regenerating liver under identical conditions of incubation, several differences were noticed (Table 1). These

Fig. 5. (a) Fractionation of [^{14}C]NAD- and [α - ^{32}P]dATP-labelled DNAase-II-solubilized pachytene chromatin on an anti-[poly(ADP-ribose)] IgG matrix; (b) polyacrylamide electrophoretic analysis of DNA in the PAC and non-PAC fractions of pachytene and regenerating liver nuclei

(a) Approx. 30 A_{260} units of pachytene nuclei were incubated with either 2 μCi of [^{14}C]NAD and unlabelled NAD (0.2 mM) or 50 μCi of [α - ^{32}P]dATP and 10 μM each of the rest of 3 dNTPS for 10 min at 25 $^{\circ}\text{C}$. They were then washed with DNAase II buffer and digested with 120 units of DNAase II to give 15% digestion. The solubilized chromatin, after adding 1 $\times$ concn. of PBS, was further fractionated on the affinity matrix as described in the Materials and methods section. $\bullet$, A_{260} absorbance; $\circ$, ^{14}C radioactivity; Δ , ^{32}P radioactivity. (b) DNA associated with various chromatin fractions were obtained by phenol/chloroform (1:1, v/v) extraction and ethanol precipitation, and 10 μg of each fraction was analysed on a 6% polyacrylamide gel and stained with ethidium bromide. Lane 1, pachytene non-PAC DNA; lane 2, pachytene PAC DNA; lane 3, pachytene undigested DNA; lane 4, regenerating liver non-PAC DNA; lane 5, regenerating liver PAC DNA; lane 6, regenerating liver undigested DNA. The numbers on the right hand side indicate the position of pBR 322 *Hinf*I markers. NS, nucleosome.

Table 1. Incorporation of [α - ^{32}P]dATP into PAC and non-PAC fractions of pachytene and regenerating liver nuclei

Incorporation of [α - ^{32}P]dATP and the subsequent immunofractionation of the DNAase II digest were carried out as described in the Materials and methods section. The DNA was purified from various chromatin fractions by proteinase K treatment followed by phenol/chloroform (1:1, v/v) extraction and ethanol precipitation. The experiment was carried out four times and the values given are specific activities of these DNA samples from a typical experiment.

Nuclei	Net radio-activity incorporated (c.p.m.)	Radio-activity released by DNAase II (%)	Specific activity of DNA (c.p.m./ μg)		
			Non-PAC DNA	PAC DNA	Undigested DNA
Pachytene	8.7×10^4	44	321	112	18
Regenerating liver	10.0×10^6	21	1266	312	621

were: (1) The net incorporation of [α - ^{32}P]dATP into these nuclei was 125-fold higher than for pachytene nuclei; (2) only 21% of the incorporated radioactivity was released by DNAase II as against 44% in pachytene nuclei; (3) the ratio of the specific activity of non-PAC DNA to that of PAC DNA was slightly higher in regenerating liver nuclei than in pachytene nuclei; and (4) the undigested DNA of regenerating liver nuclei had a much higher specific activity than that of pachytene nuclei. It is possible that the higher amount of radioactivity incorporated in the regenerating liver nuclei may reflect the number of replication sites rather than the DNA repair sites of pachytene chromatin which are much fewer in number. In addition, this may also be due

to a faster rate of the replication process compared with the DNA-repair process in the pachytene nucleus.

Pachytene PAC domains have internal strand breaks and gaps

As mentioned earlier, Malik *et al.* (1983) have shown that immunopurified PAC domains of HeLa cell chromatin had internal strand breaks. Therefore, in order to analyse whether the pachytene PAC domains also contain internal strand breaks, the DNA extracted from pachytene PAC and non-PAC fractions were labelled at their 5'-ends with [γ - ^{32}P]ATP and polynucleotide kinase. The labelled DNA was analysed on a two-dimensional gel which involved electrophoresis

Fig. 6. Analysis of internal strand breaks in pachytene PAC and non-PAC DNA by denaturing two-dimensional electrophoresis

Pachytene PAC (a) or non-PAC (b) DNA (2 μ g each) was labelled at 5'-ends with 50 μ Ci of [α - 32 P]ATP and 10 units of T4 polynucleotide kinase. The labelled DNA was subjected to 2-dimensional electrophoresis as described in the Materials and methods section. N, nucleosome.

Table 2. Labelling of PAC and non-PAC of pachytene nuclei with [γ - 32 P]ATP and polynucleotide kinase before and after calf intestinal phosphatase treatment

Phosphatased or unphosphatased pachytene PAC and non-PAC DNA (2 μ g each) were kinased with 10 units of T4 polynucleotide kinase with 50 μ Ci of [γ - 32 P]ATP as described in the Materials and methods section. The experiment was repeated three times and the total incorporation data presented are from a typical representative experiment.

Fraction	Radioactivity incorporated (c.p.m./ μ g of DNA)	
	Before phosphatase	After phosphatase
PAC	9.57×10^5	4.2×10^6
Non-PAC	1.91×10^7	2.7×10^7

in the first dimension under non-denaturing conditions and in the second dimension on a denaturing gel containing 98 % formamide. From the autoradiogram shown in Fig. 6, it is clear that while the DNA from the non-PAC fraction showed a typical diagonal distribution of radioactivity, the PAC DNA showed a substantial amount of radioactivity below the diagonal pattern in the size range of 200–400 bp revealing internal strand breaks only in the PAC DNA but not in the non-PAC DNA. In a control experiment in which the PAC DNA was subjected to electrophoresis involving non-denaturing conditions in both dimensions, there was no radioactivity below the diagonal pattern (results not shown).

While labelling the 5'-ends of pachytene PAC and non-PAC DNA with [γ - 32 P]ATP, we observed that the efficiency of labelling of non-PAC DNA was always 15–20-fold higher than that of PAC DNA (Table 2). DNAase II digestion of chromatin generates 5'-hydroxyl and 3'-phosphoryl termini in the DNA. However, if these DNA strands have to be ligated, their 5'-hydroxyl ends have to be phosphorylated, and the 3'-phosphoryl ends should be phosphatased, to serve as substrates for DNA ligase. Therefore it is likely that the pachytene PAC DNA may have some of the 5'-termini phosphorylated, and this results in a lower efficiency of labelling *in vitro* with [γ - 32 P]ATP. To see if this indeed is the situation, both the pachytene PAC and non-PAC DNA were treated with calf intestinal phosphatase before measuring labelling efficiency. The efficiency of labelling of PAC DNA after phosphatase treatment was 4.5-fold higher (Table 2), while the labelling of non-PAC DNA was increased by only 1.4-fold under identical conditions. This result clearly shows that PAC DNA has a significant number of its 5' ends phosphorylated, probably by the DNA-repair machinery associated with the PAC domains. To investigate the presence of 3'-hydroxyl ends, the Klenow fragment of *E. coli* DNA polymerase I was used, which adds 5'-nucleotides to 3'-hydroxyl ends, fills the gaps, if any, and lacks 5'-to-3'-exonuclease activity. To our surprise, substantial incorporation of [α - 32 P]dATP was observed into PAC DNA (Table 3). Under identical conditions, incorporation of [α - 32 P]dATP into non-PAC DNA was 22-fold lower than incorporation into PAC DNA. In order to find out whether the presence of gaps in the pachytene PAC DNA is a

Table 3. Gap filling of pachytene PAC DNA with Klenow fragment of DNA polymerase I

The reaction was carried out as described in the Materials and methods section. The experiment was carried out three times and the results presented are from a typical experiment. Gap size/DNA molecule was measured assuming that all four dNTPs contributed equally to the gap-filling reaction.

Fraction	$\pm$ NAD	Radioactivity incorporated (c.p.m./0.5 μ g of DNA)	DNA gap size (bases)	
			Per 200 bp DNA molecule	Per 400 bp DNA molecule
Pachytene PAC	+	2.8×10^7	23	12
Pachytene non-PAC	+	1.3×10^6	1	11
Pachytene PAC	—	1.8×10^7	15	8
Regenerating liver PAC	+	2.4×10^6	2	1

Fig. 7. Thermal denaturation of DNA samples isolated from various chromatin fractions

DNA samples were in $0.1 \times \text{SSC}$. Curve 1, rat DNA (32.5); 2, gap-filled and ligated pachytene PAC DNA (26.4); 3, pachytene non-PAC DNA (26.2); 4, regenerating liver PAC DNA (22.2); 5, pachytene PAC DNA (13.2).

general phenomenon associated with poly(ADP-ribosyl)-ation, or whether the gaps are related to a DNA-repair process specific to pachytene PAC DNA, the experiment was repeated with the regenerating liver chromatin DNA fractions under identical conditions (Table 3). Although the net incorporation into regenerating liver PAC DNA was slightly higher than that into pachytene non-PAC DNA, it was 12-fold lower than that into pachytene PAC DNA.

The presence of gaps in the pachytene PAC DNA was further confirmed by thermal denaturation analysis of DNA. Fig. 7 shows the hyperchromicity profiles of DNA isolated from various chromatin fractions.

Rat genomic DNA isolated from rat liver nuclei showed 32.5% hyperchromicity with a T_m value of 70.5°C. On the other hand, pachytene PAC DNA showed only 13.2% hyperchromicity, indicating that there are substantial numbers of single-stranded DNA regions. This confirms the results obtained by gap filling (Table 3). The pachytene non-PAC DNA, however, showed 26.2% hyperchromicity. The pachytene PAC DNA, after gap filling with the Klenow enzyme in the presence of all four cold dNTPS and DNA ligation, showed 26.12% hyperchromicity. The regenerating liver PAC DNA showed a hyperchromicity of 22.2%. The gap detected by the thermal denaturation technique seems to be much greater than that calculated with data obtained by gap filling with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$. It is possible that the latter case represents a lower limit, as only one radioactive nucleotide was used for the gap-filling assay. These results, therefore, strengthen our earlier conclusion that the gaps and internal strand-breaks observed in pachytene PAC DNA are characteristic of the pachytene nucleus, most probably due to the ongoing DNA-repair process, and are not a general feature of PAC domains isolated from other types of cells.

Fig. 8. DNA dot-blot analysis of PAC and non-PAC DNA of pachytene and regenerating liver

(a) DNA samples from different fractions were spotted on to nitrocellulose sheet and hybridized with nick-translated pachytene PAC DNA. Lane a, regenerating liver non-PAC; b, regenerating liver PAC; c, regenerating liver DNAase II undigested DNA; d, pachytene non-PAC DNA; e, pachytene PAC DNA; f, pachytene DNAase II undigested DNA. (b) DNA samples (100 ng) were spotted on to nitrocellulose sheets and hybridization and high stringent washings were carried out as described in the Materials and methods section except that the probe was pachytene PAC DNA (isolated from nuclei without preincubation with NAD) and gap filled with Klenow polymerase in the presence of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$. Lane a, pachytene PAC DNA obtained from micrococcal-nuclease-solubilized chromatin (24% digestion); b, pachytene PAC DNA obtained from the DNAase II digest of nuclei preincubated with NAD; c, pachytene PAC DNA obtained from DNAase II digest of nuclei without preincubation with NAD.

Strand breaks and gaps in pachytene PAC DNA represent the picture *in vivo* and are not artefacts due to the isolation procedure

In the studies described above, the nuclei were preincubated with NAD for the isolation of PAC domains mainly to suppress poly(ADP-ribose) glycohydrolase (Malik *et al.*, 1983). One can argue that nicks are created within the DNA duplex during this incubation period by the action of endogenous nucleases, which in turn stimulates the poly(ADP-ribosyl)ation of chromatin proteins at these nicked sites. However, several arguments do not support this possibility. First, when the digestion of pachytene chromatin was carried out with DNAase II without prior incubation with NAD, there was no change in the percentage of pachytene chromatin bound to the affinity column. Secondly, the gap-filling experiments carried out with pachytene PAC DNA isolated without prior incubation with NAD showed that substantial gaps were still present within the DNA (Table 3). Thirdly, when the DNA sequences associated with pachytene PAC DNA isolated with and without prior incubation with NAD were compared by dot-blot hybridization, the intensity of the signal was very similar (Fig. 8). Finally, we would also like to stress here that DNAase II was preferred over micrococcal nuclease in the present studies to solubilize the chromatin since the digestion could be carried out in the absence of any divalent cations and in the presence of 0.25 mM-EDTA. For comparative analysis we have also isolated pachytene PAC domains after micrococcal nuclease digestion, and the DNA sequences of these domains were compared with pachytene PAC DNA obtained after DNAase II solubilization. As can be seen from Fig. 8(b), there was no difference in the hybridization signal between these two DNA sequences. Thus, these results together with the distinct characteristics of pachytene PAC DNA as compared with regenerating liver PAC DNA clearly show that the characteristics of pachytene PAC DNA indeed represent the situation *in vivo* and are not artefacts of the experimental conditions.

DNA sequences associated with PAC DNA of pachytene and regenerating liver are different

To answer the question whether PAC DNA sequences of regenerating liver and pachytene show any similarity, DNA associated with the different chromatin fractions was analysed by dot-blot analysis using nick-translated pachytene PAC DNA as the probe (Fig. 8a). A hybridization signal was seen with both pachytene non-PAC DNA and the DNA associated with undigested chromatin. As can be expected, the probe hybridized strongly with the homologous pachytene PAC DNA. A more intriguing observation is that while the pachytene PAC-DNA probe hybridized with the non-PAC DNA and undigested chromatin of regenerating liver, there was no hybridization signal with PAC DNA of regenerating liver. From these results, it can be concluded that the DNA sequences associated with the PAC domains of pachytene and regenerating liver are different. The PAC domains of regenerating liver may represent the DNA sequences of origins of replication and nearby regions which are actively undergoing replication at 18 h (Jump *et al.*, 1979). The observation that pachytene DNA did hybridize with pachytene non-PAC and undigested DNA is rather intriguing, and it is possible

Fig. 9. DNA dot-blot analysis of fractionated pachytene chromatin using total cDNA probe

Different concentrations of various DNA samples were spotted on to nitrocellulose sheets and then hybridized with [α - 32 P]dATP-labelled pachytene cDNA synthesized against cytoplasmic poly(A⁺)RNA as described in the Materials and methods section. Lane a, non-PAC DNA; b, PAC DNA, and c, undigested pellet DNA.

that part of the PAC DNA may represent non-unique-type DNA sequences.

Association of poly(A⁺) RNA coding sequences in the pachytene PAC domains

Pachytene spermatocyte nuclei are also active in RNA synthesis (Kleene *et al.*, 1983). To study the distribution of mRNA coding sequences among the various chromatin domains, dot-blot hybridization was carried out using a labelled cDNA probe derived from cytoplasmic poly(A⁺) RNA of pachytene cells. It can be seen from Fig. 9 that all the three fractions (PAC, non-PAC and pellet) gave the signal. However, the hybridization signal with the non-PAC fraction was significantly greater than with the DNA of PAC and pellet fractions. Based on the observation that approx. 14% of the DNAase-II-solubilized chromatin bound to the affinity column, it can be calculated that the association of messenger coding sequences is at least 7-fold higher in the non-PAC fraction than in the PAC fraction. Analysis of the ongoing transcriptional activity indicated that the specific radioactivity of the non-PAC fraction was also approx. 8-fold higher than that of the PAC fraction (Table 4).

Polypeptides associated with PAC and non-PAC domains of pachytene and regenerating liver

The proteins associated with PAC and non-PAC domains of pachytene and regenerating liver nuclei were analysed on an SDS/polyacrylamide gel (5–15% gradient). The pattern shown in Fig. 10 reveals that, although the same amounts of chromatin were used to analyse the protein, the PAC fractions had comparatively less histone content than the non-PAC fractions. A comparison of the PAC and non-PAC lanes of pachytene nuclei reveals that seven non-histone proteins are enriched in the PAC lane that are absent in the non-PAC lane. Among these polypeptides, six in the molecular mass range of 50–75 kDa are represented in the PAC lane of regenerating liver, while the 20 kDa protein is totally absent (see lanes d and f). While the non-PAC fraction of pachytene nuclei did not show any non-histone proteins, there were eight polypeptides in the molecular mass range of 45–80 kDa in the non-PAC fraction from regenerating liver. Another

Fig. 10. Analysis of polypeptides associated with PAC and non-PAC fractions of pachytene and regenerating liver

Different chromatin fractions (4 A_{260} units in each case) were digested with DNAase II (10 μ g) and processed as described in the Materials and methods section. Polypeptides were analysed on an SDS 5–15% gradient polyacrylamide gel. The polypeptides were revealed by Coomassie Blue staining. Lane a, molecular mass markers (conalbumin, 80 kDa; bovine serum albumin, 68 kDa; ovalbumin, 45 kDa and cytochrome c, 12 kDa); b, pancreatic DNAase I; c, pachytene non-PAC; d, pachytene PAC; e, regenerating liver non-PAC; f, regenerating liver PAC; g, total testicular histones. Arrows in lane d indicate faint bands.

interesting observation that can be made from the gel pattern is that while the pachytene non-PAC fraction did not show any stainable histone H1t (testis-specific histone H1 subtype), the corresponding PAC fraction showed a

distinct histone H1t band suggesting that this testis-specific histone variant is enriched in the poly(ADP-ribosyl)ated chromatin domains of the pachytene nucleus. The band corresponding to histone H1t has indeed been shown to be histone H1t, as indicated by immunoblot analysis using highly specific polyclonal antibodies against histone H1t (Markose & Rao 1985; results not shown).

DISCUSSION

The present investigation was aimed at isolating and characterizing the poly(ADP-ribosyl)ated chromatin domains of pachytene spermatocytes in view of the fact that poly(ADP-ribosyl)ation of chromatin proteins is closely correlated with DNA-repair activity (Gaal & Pearson, 1985), and the only DNA-synthetic activity in the pachytene nucleus is related to recombination-linked DNA-repair events (Stern & Hotta, 1987). In fact, one of the earlier studies had shown that poly(ADP-ribosyl)ation of nuclear proteins is much higher in the mouse testis than in the liver (Faraone-Menella *et al.*, 1982). Subsequently, Coromines & Mezquita (1985) showed that a mixture of premeiotic and meiotic germ cells of rooster testis had a much higher poly(ADP-ribose) polymerase activity than postmeiotic round spermatids. The results presented in this paper on both immunofluorescent staining as well as [14 C]NAD incorporation have further shown that the rate of poly(ADP-ribosyl)ation of rat pachytene nuclear proteins is several-fold higher than in either premeiotic or postmeiotic germ cell nuclei.

The immunopurified pachytene poly(ADP-ribosyl)ated chromatin domains show several interesting molecular features. The presence of internal strand breaks in the pachytene PAC domains is shown. More interestingly, the presence of a substantial gap in the pachytene PAC DNA [as shown by gap filling with Klenow polymerase (Table 3) as well as by thermal denaturation analysis] which is absent in the regenerating liver PAC DNA, indicates that this gap is very specific to pachytene DNA metabolism and is not linked with the phenomenon of poly(ADP-ribosyl)ation itself.

Analysis of the polypeptides associated with various chromatin fractions has revealed several interesting features. First, many of the non-histone proteins in the PAC domains of both pachytene and regenerating liver are very similar (Fig. 10), although the DNA sequences associated with them were quite different (Fig. 8a). These polypeptides may represent common proteins/enzymes

Table 4. Association of ongoing transcriptional activity in fractionated pachytene PAC and non-PAC domains

Transcription was carried out *in vitro* by incubating 20 A_{260} units of pachytene nuclei with 100 μ Ci (experiment I) or 50 μ Ci (experiment II) of [α - 32 P]UTP in a total volume of 1.5 ml for 30 min, after which the nuclei were digested with DNAase II to give 8% digestion. The free label in the digest was removed on a column of Sephadex G-50 and subsequently fractionated on the anti-[poly(ADP-ribose)] IgG affinity column.

Experiment	Total incorporation (c.p.m.)		Non-PAC/ PAC	Specific activity (c.p.m./ μ g of DNA)		Non-PAC/ PAC
	PAC	Non-PAC		PAC	Non-PAC	
I	15480	139288	9.0	447	3715	8.3
II	3734	38448	10.3	120	898	7.5

involved in the two closely related processes. Secondly, a 20 kDa polypeptide present in the pachytene PAC domains was absent in the PAC domains of regenerating liver. The molecular size of this protein does not correspond to any of the reported meiosis-specific proteins (Hotta & Stern, 1981; Hotta *et al.*, 1985), and it is quite likely that it may play an important role in the recombination-linked DNA-repair process of the pachytene nucleus. Thirdly, the testis-specific histone H1t was enriched in the pachytene PAC domains compared with the non-PAC domains. Recently, it has been demonstrated that poly(ADP-ribosylation) of histone H1 results in relaxation of the higher order structure of chromatin (deMurcia *et al.*, 1986). Fig. 4 shows that histones TH1 in Triton X-100-acid-urea gel comprising histones H1a and H1t were poly(ADP-ribosylated). Furthermore, we have recently shown that histone H1t is antigenically distinct among the histone H1 subtypes and had speculated that altered structural domains of histone H1t could modulate chromatin structure to facilitate the meiotic events. In this connection, the enrichment of histone H1t in the PAC domains containing the DNA-repair domains assumes great significance.

Poly(ADP-ribosylation) of chromatin proteins has also been suggested to play a role in gene expression (Gaal & Pearson, 1985; Ueda & Hayashi, 1985). However, there are also reports contradicting this idea and one study has not shown any preferential association of transcriptional activity with the poly(ADP-ribosylated) chromatin domains (Hough & Smulson, 1984). Furthermore, poly(ADP-ribosylated) histones act as potent suppressors of transcription *in vitro*, and the site of inhibition was shown to be on the template and not RNA polymerase (Fu-Liyu, 1985). The results of the present investigation show that only 10% of the mRNA coding sequences present in the DNAase-II-solubilized chromatin (at 15% digestion) are associated with pachytene PAC domains and hence do not favour the correlation between poly(ADP-ribosylation) and transcriptional activity. At the same time, however, it is clear that the PAC domains contain not only DNA-repair domains but also some of the transcribing chromatin as well. But the observations that (1) strand breaks and gaps were present only in the pachytene PAC fraction and not in the non-PAC fraction and (2) 90% of the transcribing chromatin domains were associated with the non-PAC fraction, suggest that the DNA-repair domains are highly enriched in the pachytene PAC fraction.

Several of the molecular properties of the pachytene PAC DNA resemble the characteristics of pachytene DNA metabolism observed both in *Lilium* and in mouse (Stern & Hotta, 1987). Therefore, it is tempting to speculate that the DNA-repair domains present in the pachytene PAC domains may represent the sites of meiotic recombination between homologous chromosomes. However, we would like to stress that the conclusions drawn here are based on labelling of pachytene chromatin *in vitro* with [α - 32 P]dATP and fractionation of chromatin domains on an anti-[poly(ADP-ribose)] IgG affinity column. Therefore, further confirmation that PAC domains do indeed contain the repair domains linked to recombination should come from studies *in vivo*.

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REFERENCES

- Alfageme, C. R., Zweidler, A., Mahowald, A. & Cohen, L. H. (1974) *J. Biol. Chem.* **249**, 3729–3736
- Coromines, M. & Mezquita (1985) *J. Biol. Chem.* **260**, 16269–16273
- deMurcia, G., Huletsky, A., Lamarre, D., Gaureadu, A., Pouyet, J., Daune, M. & Poirier, G. G. (1986) *J. Biol. Chem.* **261**, 7011–7017
- Faraone-Menella, M. R., Quesada, P., Farina, B., Leone, E. & Jones, R. (1982) *Biochem. J.* **205**, 245–248
- Fu-Liyu (1985) *FEBS Lett.* **190**, 109–114
- Gaal, J. C. & Pearson, C. K. (1985) *Biochem. J.* **230**, 1–18
- Gold, B., Stern, L., Bradley, F. M. & Hecht, N. B. (1983) *J. Exp. Zool.* **225**, 123–134
- Guertin, M., Baril, P., Bartkowiak, J., Anderson, A. & Belanger, L. (1983) *Biochemistry* **22**, 4296–4301
- Hotta, Y. & Stern, H. (1981) *Cell (Cambridge, Mass.)* **27**, 309–319
- Hotta, Y., Tabata, R. A., Bouchard, R. A., Pinon, R. & Stern, H. (1985) *Chromosoma* **93**, 140–151
- Hough, J. C. & Smulson, M. E. (1984) *Biochemistry* **23**, 5106–5023
- Jump, O. J., Butt, T. R. & Smulson, M. E. (1979) *Biochemistry* **18**, 983–990
- Kanai, Y., Miwa, M., Matsushima, T. & Sugimura, T. (1974) *Biochem. Biophys. Res. Commun.* **59**, 300–306
- Kleene, K. C., Distel, R. J. & Hecht, N. B. (1983) *Dev. Biol.* **98**, 455–464
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Livingston, M. D. (1974) *Methods Enzymol.* **34B**, 723–731
- Malik, N., Miwa, M., Sugimura, T. & Smulson, M. E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2554–2558
- Maniatis, T., Jeffrey, A. & van de Sande, H. (1975) *Biochemistry* **14**, 3787–3794
- Maniatis, T., Fritsch, E. E. & Sambrook, T. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, New York
- Markose, E. R. & Rao, M. R. S. (1985) *J. Biol. Chem.* **260**, 16263–16268
- Meinkoth, J. & Wahl, G. (1984) *Anal. Biochem.* **138**, 267–284
- Ouchterlony, O. (1958) *Progr. Allergy* **5**, 1–45
- Rao, M. R. S., Rao, B. J. & Ganguly, J. (1982) *Biochem. J.* **205**, 15–21
- Rao, B. J., Brahmachari, S. K. & Rao, M. R. S. (1983) *J. Biol. Chem.* **258**, 13478–13485
- Rill, R. L., Ramsay-Shaw, B. & Van Holde, K. E. (1978) *Methods Cell Biol.* **18**, 69–103
- Shima, T., Fuzimura, S., Hasegawa, S., Shimizu, Y. & Sugimura, T. (1970) *J. Biol. Chem.* **245**, 1327–1330
- Smulson, M. E. & Sugimura, T. (1984) *Methods Enzymol.* **106**, 438–440
- Stern, H. & Hotta, Y. (1980) *Mol. Cell. Biochem.* **29**, 145–158
- Stern, H. & Hotta, Y. (1987) in *Meiosis* (P. B. Moens, ed.), pp. 303–331, Academic Press, New York
- Stubbs, L. & Stern, H. (1986) *Chromosoma* **93**, 529–536
- Sugimura, T., Yoshimura, N., Miwa, M., Nagari, H. & Nagao, M. (1971) *Arch. Biochem. Biophys.* **147**, 660–665
- Ueda, K. & Hayashi, D. (1985) *Annu. Rev. Biochem.* **54**, 73–100
- Yoshihara, K., Itaya, A., Tanaka, Y., Ohashi, Y., Ito, K., Teroka, H., Tsukaola, K., Matsukage, A. & Kamiya, T. (1985) *Biochem. Biophys. Res. Commun.* **128**, 61–67