

# Poly (ADP-ribosyl)ation as one of the molecular events that accompany mammalian spermatogenesis.

**Piera Quesada**

Department of Organic and Biological Chemistry, University Federico II of Naples; via Mezzocannone 16, 80134 Napoli, Italy.

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Correspondence: Piera Quesada: Phone +39-81-7041235, Fax: +39-81-5521217, E-mail [quesada@unina.it](mailto:quesada@unina.it)

## Summary

It is known that mammalian spermatogenesis is a synchronous process of cellular differentiation during which morphological changes occur, concomitantly with alterations in the complement of constituent proteins, that reflect differences in the mRNA populations coding for stage-specific proteins. Moreover, the most dramatic changes in chromatin structure observed in eukaryotes, take place during spermiogenesis, and the main nuclear processes occur in well-defined cell stages. Rat testis has been used as experimental model in a research project carried out at various levels, represented by rat germinal cells (primary and secondary spermatocytes, round spermatids), chromatin fractions (transcriptionally active chromatin, nuclear matrix, MARS) and purified nuclear proteins (histone and non-histone proteins). Specific experiments have been carried out in order to determine the poly(ADPR)polymerase content at different stages of germ-cell differentiation, the poly(ADP-ribose) amount, length and complexity inside the nucleus, and the poly(ADP-ribose) acceptors among tissue- and stage-specific nuclear proteins. The results indicate that regulation of the poly(ADPribosyl)ation system accompanies the earlier phases of the germinal cell differentiation. Indeed, poly(ADPribose)polymerase is particularly active in primary spermatocytes, being possibly implicated in the recombination events that characterize the pachytene phase of the meiotic division. Different classes of poly(ADPribose) modify different chromatin fractions (DNase I-sensitive, DNase I-resistant chromatin, and nuclear matrix) implicated in DNA replication, repair and transcription. Moreover, the H1 variant H1t, specifically expressed in pachytene spermatocytes, represents the main poly(ADPribose) acceptor, together with poly(ADPR)polymerase itself, in rat germ-cells. Its modification can amplify the role of histone H1 variants as modulators of chromatin structure.

## I. Introduction

### A. Overview of mammalian spermatogenesis

Mammalian spermatogenesis is a highly specialized process of differentiation and one of the most dramatic events that any single cell manifests. It is a continuous developmental process that occurs in the adult, and it offers unique opportunity to study the regulation and execution of cell differentiation programs.

As a result of years of work by many expert biologists a detailed histological description of the process is available; the endocrine and paracrine hormonal control has been assessed. However, very little is still known about the molecular and cellular events that underlie spermatogenesis. Such evidences can now be achieved applying the powerful tools of molecular genetics to this important area of biology (Meiestrich, 1993).

Spermatogenesis is composed of three major phases: (i) mitotic proliferation of spermatogonia, (ii) two reductional divisions of meiosis that produce the haploid

**Figure 1.** Schematic representation of the different populations of germinal cells which characterize the different stages of spermatogenesis. The nucleoproteins present during various stages of spermatogenesis are correlated with cell morphology.

spermatids, and (iii) extensive remodelling of these cells during spermiogenesis to form the mature spermatozoa (**Figure 1**).

Because cytokinesis is incomplete at each of the mitotic and meiotic cell divisions, descendants of a single stem cell develop within a syncytium in which cells are connected by intracellular bridges (Braun et al., 1995). It has been proposed that the cytoplasmic bridges allow the passage of various macromolecules between cells, thus ensuring synchronous development of all cells within a clone, and gametic equivalence between haploid spermatids.

The modification of the genome that occurs during spermatogenesis establishes the pattern of paternal expression that is essential for successful embryonic development and the normal phenotype of the adult (Meistrich, 1993).

## **B. Meiosis and recombination**

Of the three phases of the spermatogenesis (mitotic proliferation, meiosis and post-meiotic differentiation) meiosis most closely defines gametogenesis since no mammalian cell other than germ cells undergo meiosis. Moreover, in female gametocytes the meiosis occurs during the fetal life, whereas in male gametocytes this process occurs during the adult life and can be more easily studied.

The unique events of meiosis include the pairing and recombination of chromosomes during prophase of meiosis I, and the segregation of homologous chromosomes during anaphase of meiosis I. In meiotic

prophase chromosomes must find their homologues, establish and maintain pairing, and they must carry out the steps of recombination. Later on, chromosomes undergo condensation, and recombination events are resolved as crossover, that in the metaphase, stabilize the alignment of homologues at the spindle equator, thus ensuring regular segregation of homologous chromosomes from one another during the ensuing anaphase (Hawley, 1988).

All these events are essential for production of chromosomally balanced gametes; errors in pairing or recombination in meiotic prophase lead to errors in anaphase I segregation, giving rise to aneuploid offspring by the production of chromosomally unbalanced gametes. Mutagens, in particular aneuploidogens and clastogens, can induce such errors. To understand the etiology of chromosomally abnormal gametes, the knowledge of the normal mechanisms of chromosome behaviour during meiosis is required.

Cell-cycle check-points describe the mechanism by which cells assess the completion of events required for successful progress through the cell cycle and production of euploid cell products (Hartwell and Weinert, 1989). There may be a number of spermatogenesis meiosis checkpoints and these may assess both completion of recombination and accumulation of precursors for spermiogenesis.

For instance, it is not known what happens during pachytene and why it is so lengthy. The supposition might previously have been that the process takes days because this much time is needed to carry out the genetic events of recombination. However it has been demonstrated that the recombination events have reached the point when

chiasmata can be formed (Collins and Newton, 1994). Thus, it may be not the genetic events that account for the length of time that the spermatocyte spends in this phase of its differentiation. Instead, the length of pachytene may be determined by the need to synthesize precursors that will be used for post-meiotic spermiogenesis.

Different kinds of pairing occur during meiosis, which involve base-pairing (DNA-DNA) interactions. Recently an alternative model has been presented based upon protein-DNA interactions involved in transcription (Cook, 1997). Interestingly, the condensation of chromatin into discernible chromosomes usually inhibits transcription. In contrast the "aligned" chromosomes during meiotic prophase I are transcriptionally active. Indeed, it is remarkable the observation that chromosomes only pair when they are transcriptionally active. A general model has been described for pairing based upon promoter-polymerase interactions on the light of the observation that each chromosome in the haploid set has a unique array of transcription units. Indeed a correct pairing would be nucleated when a promoter binds productively to a homologous site in another transcription factory and is then the consequence of transcription of partially condensed chromosomes.

A concept is emerging that multiple components of nuclear organization contribute to competence for gene expression. Chromatin structure, nucleosome organization and gene-nuclear matrix interactions provide a basis for rendering sequences accessible to transcription factors, supporting integration of activities at independent promoter elements of cell cycle and tissue specific genes.

The structure of the eukaryotic nucleus is still an area of interest in cell biology; it is well established that both interphase chromatin and mitotic chromosomes are organized into loops, anchored to a nuclear matrix by specific DNA sequence landmarks named MARs (matrix associated regions) or SARs (scaffold associated regions) (Boulikas, 1993a). Such DNA sequences have been demonstrated to contain origins of replication and transcription enhancers (Jackson and Cook, 1985; Jackson and Cook, 1986); then it has been suggested that the nuclear matrix may play a key role in genome organization and gene potentiation. For instance, the common structural features of replication origins in all life forms have been assessed (Boulikas, 1996); this knowledge is of importance for the understanding of the mechanisms underlying the differential expression of genes that colocalize with matrix associated regions.

As in the somatic nucleus, chromatin within the male gametes is organized in discrete loops; however, these loops differ from their somatic counterparts with respect to the packaging of their DNA and their average size. Loops within the sperm nucleus are approx. 27Kb in size (compared to 60Kb in somatic cell nuclei). The sperm nuclear matrix attachment regions (SMARs) show a

somatic-like organization; furthermore, it has been demonstrated that a specific subset of haploid specific and constitutively expressed genes are associated with the sperm nuclear matrix (Kramer and Krawets, 1996); thus the mature sperm genome is organized in a specific non-random manner.

### **C. Chromatin structure and function in differentiating germinal cells**

The most dramatic changes in chromatin structure and function observed in eukaryotes take place during spermatogenesis: (i) DNA replication occurs in spermatogonia and prior meiosis in preleptotene spermatocytes. Spermatocytes undergo meiosis producing spermatids which no longer divide but differentiate into mature spermatozoa; (ii) spermatogonia, spermatocytes, and early spermatids are active in nuclear transcription, whereas spermatids undergoing differentiation as well as spermatozoa are totally inactive; (iii) an interval of regulated DNA nicking followed by repair synthesis occurs in meiotic cells at pachytene phase (DNA recombination); (iv) late spermatids are genetically inactive in DNA replication and transcription; however, these cells are still able to repair their genetic damage before the final nuclear condensation of chromatin occurs; (v) the chromatin of spermatids undergoing differentiation to spermatozoa becomes relaxed in late spermatids, exposing binding sites on DNA at regions of nucleosome disassembly.

Cell type-specific expression of genes encoding these germ-line nuclear proteins may be regulated at the transcriptional level and/or at the post-transcriptional level.

Differences in post-transcriptional processing have been observed during spermatogenesis. Stabilization of mRNA via polyadenylation and the increased efficiency of translational reinitiation associated with this process may be critical. In this context, the replication-independent histone mRNAs that are restricted to specific cell types (e.g. those encoding H5, the H1 variant in nucleated red blood cells) are polyadenylated, while the short-lived replication dependent histone transcripts do not contain poly(A) tracts. The polyadenylated histone mRNA lacks the conserved terminal hairpin structure seen in non-polyadenylated transcripts and instead contains the (polyA) addition sequence (AAUAAA) upstream of the poly(A) tract.

The rat testis-specific TH2b histone gene assumes a hypomethylated chromatin structure at all stages of spermatogenesis. The H1t mRNA level rises sharply in meiotic pachytene spermatocytes, being very low in pre-meiotic spermatogenic cells as a result of transcriptional repression of the gene by a pre-meiotic cell-specific protein. A temporal correlation has been observed between

the appearance of testis specific DNA binding proteins and the onset of transcription of the testis-specific histone H1t gene (Grimes et al., 1992).

The appearance of stage-specific mRNA during spermatogenesis has been demonstrated (Thomas et al., 1989). Experiments involving in vitro translation of mRNA from isolated germ cells have suggested that transcription of the phosphoglycerate kinase-2 (PGK-2) and lactate dehydrogenase (LDH-C) genes first occurs in pachytene spermatocytes and continues in round spermatids. The expression of two DNA repair related enzymes poly(ADPR)polymerase and DNA polymerase  $\beta$ , also varies in germinal cells. While the spermatocytes were shown to contain both enzymes as well as their transcripts, in other cell types this has not been observed (Menegazzi et al., 1991).

Screening of testis cDNA libraries have identified cDNA clones that are transcribed specifically by round spermatids, such as the protamines (Thomas et al., 1989). Developmentally-regulated patterns of gene expression have been explored and the data suggest the following: (i) stage-specific patterns of transcription occur coincidentally with the appearance and accumulation of distinct germ cell types within the seminiferous epithelium; (ii) transcription of a variety of genes occurs exclusively within haploid spermatids, while specific transcripts accumulate in spermatogonia and meiotic germ cells but not in spermatids; (iii) several transcripts are detected initially during meiotic prophase and continue to accumulate in round spermatids; (iv) several multigene families express germ cell-specific isotypes, including  $\alpha$ -tubulin,  $\beta$ -actin, LDH and PGK; (v) various oncogenes are expressed in germ cells.

Collectively, these observations suggest that precise controls exist for determining stage specific gene expression during spermiogenesis (Erikson, 1990).

The differentiation of round spermatids into mature spermatozoa requires the synthesis of hundreds of new proteins and the assembly of a unique collection of organelles. During this differentiation process a flagellum is constructed the acrosomal vesicle is formed and the nucleus is compacted to approximately one-tenth of its volume (Hecht, 1986).

Spermiogenesis is characterized by chromatin condensation and the replacement of histones typical of earlier spermatogenic cells by the highly basic protamines. During this period the nucleosomal pattern of somatic chromatin is lost in round spermatids and replaced by an highly compacted structure in mature sperm. The replacement of histones involves the elimination of somatic and germ cell specific histones. Variants for histone H1, H2a, H2b and H3 that appear during the mitotic and meiotic phases of spermatogenesis have been described in both mouse and rat germinal cells (Meistrich, 1989).

Several reports suggested that histone synthesis ceases during spermiogenesis as these proteins are replaced by a set of transitional proteins (TPs) in both rat and mouse. Later in spermiogenesis the TPs are replaced by protamines which persist in mature sperm. While the organization of sperm chromatin is unknown, the appearance of germ cell-specific histone variants, TPs and protamines suggest that multiple proteins are involved in chromatin remodelling.

The detailed mechanisms by which nucleosomes are disassembled and the DNA finally compacted during spermiogenesis remain unknown. Several factors affect this mechanism including post-translational modification of histones. A correlation exists between the occurrence of extensive H4 acetylation in spermatids and the presence of protamines in spermatozoa (Meistrich, et al. 1992). Acetylation of specific lysines, as well as other kind of post-translational modification, can reduce protein positive charge which is believed to modify their interaction with DNA (Oliva et al., 1987)

Besides acetylation, phosphorylation and methylation, ADPRibosylation also plays a fundamental role in gene regulation as it can dramatically influence DNA/histone interactions, particularly during cell differentiation (Lautier et al., 1993).

In recent years the elucidation of the molecular mechanisms of the poly(ADPR)ation reactions has advanced rapidly. The nuclear enzyme poly(ADPR)polymerase (**PARP**) participates in several nuclear events (DNA replication, transcription and repair) by catalyzing the post-translational modification of chromosomal proteins. After its activation by DNA strand breaks, this enzyme catalyzes its self-modification and the modification of other DNA binding proteins with variably sized ADPRibose polymers (**pADPR**) consuming the nuclear pool of  $\beta$ -NAD (Althaus and Richter, 1987).

pADPR is a homopolymer made up of adenosine diphosphate ribose units. The nature of this polymer is now well characterized as structurally similar to a nucleic acid. Branching points up to a proportion of 3% can be found within the polymer, contrary to DNA or RNA. ADPRibose polymers vary in complexity and may reach a length of more than 200 residues. The half-life of pADPR varies in relation to the length of the polymer chain as well as to the nature of the acceptor protein: after stimulation of pADPR synthesis with alkylating agents, the half-life of pADPR is less than 1 min. The very short life of pADPR suggests the presence of very high polymer turnover in intact cells.

Indeed, many enzymes are involved in pADPR metabolism. The poly(ADPR)polymerase, responsible for the synthesis of the pADPR, catalyzes the initiation, elongation and branching steps. PARP has also an abortive NADase activity, with a  $K_m$  four times that of the polymerase activity. The elongation mode is probably

distal and is accomplished in a distributive fashion with respect to the acceptor (Mendoza-Alvarez and Alvarez-Gonzales, 1993).

pADPR catabolism in cells is achieved by three different enzymes the poly(ADPR)glycohydrolase (Hatakeiama, et al. 1986 ) the ADPRibosyl protein lyase (Okayama et al. 1978) and a phosphodiesterase that breaks the pyrophosphate moieties of pADPR (Futai and Mizuno, 1967). The action of poly(ADPR)glycohydrolase (**PARG**) is the most important and catalyzes polymer degradation by exoglycosidic hydrolysis following an endoglycosidic incision. Two different forms have been found in many tissues: the existence of a nuclear and a cytoplasmic pADPR glycohydrolase has been suggested. Various factors modulate pADPR glycohydrolase activity including the nature of the acceptors and the length of the polymer (Hatakeiama, et al. 1986 )

#### **D. Poly(ADPRibosyl)ation reactions during rat germinal cell differentiation**

In order to define the role of the poly(ADPRibosyl)ation system during germinal cell differentiation, we have used the rat testis as an experimental model. Our investigations have been carried out at various levels: (i) at the cellular level using different rat germinal cells (primary and secondary spermatocytes, haploid spermatids); (ii) at the nuclear level using isolated chromatin fractions (transcriptionally active chromatin, nuclear matrix, MARs); (iii) at the protein level using purified nuclear proteins (histone and non-histone proteins).

In addition, we have carried out specific experiments in order to determine: (i) the content and enzymatic activity of poly(ADPR)polymerase at different stages of germ-cell differentiation; (ii) the amount, length and complexity of poly(ADPRibose); (iii) the poly(ADPRibose) acceptors among tissue- and stage-specific nuclear proteins.

##### **1. Cellular level**

There is a great deal of evidence advocating for a role of PARP in the propagation of epigenetic information. PARP activity correlates with the species-specific life span among mammalian species (Grube and Burkle, 1992). Malignant transformation has been correlated to changes in the regulation and expression of genes caused by poly(ADPRibosyl)ation dysfunction (Borek and Cleaver, 1986). The expression level of the PARP gene varies during differentiation in several cell lines (Smulson et al., 1995) and, in general, PARP seems to be more active in the S and G2 phases of the cell cycle (Leduc et al., 1988).

Moreover, it has been inferred that pADPR metabolism is involved mainly in base excision repair (Mathis and Althaus, 1990; Satoh and Lindahl, 1992;

Malanga and Althaus, 1994; Lindahl et al., 1995). Differences in PARP activity can be interpreted in many ways: evidence has been obtained for changes in the pADPR synthesis pattern (Satoh et al., 1994), and in the nature of the poly(ADPRibosyl)ated proteins (Boulikas, 1990), as a function of particular chromatin structure activities.

A functional correlation was observed between early phases of spermatogenesis and poly(ADPRibosyl)ation in the rat (Quesada et al., 1996).

##### **2. Nuclear level**

It is now possible to have a better insight into the internal structure of the interphase nucleus. The nuclear framework is now characterized as a non-histone protein scaffold supporting the attachment points of DNA loops (Jack and Eggert, 1992), this structure seems to influence gene replication and transcriptional activity. It is widely accepted that DNA replication and transcription (Jackson and Cook, 1985; Jackson and Cook, 1986) as well as DNA repair (Mc Cready and Cook, 1984) are actively coordinated by the nuclear matrix.

It is thought that PARP plays a role in the maintenance of genetic integrity. As an example, PARP is defective in the cells from patients suffering from xeroderma pigmentosum, who are unable to excise pyrimidine dimers induced by ultraviolet radiation (Wood et al. 1988).

A possible mechanism for the involvement of the poly(ADPRibosyl)ation reaction, in different chromatin functions has been proposed (Realini and Althaus, 1992) that takes into account all the described features of the poly(ADPRibosyl)ation reaction. According to this mechanism, histone proteins are reversibly detached from the chromatin by the concerted action of poly(ADPR)polymerase and poly(ADPR)glycohydrolase; the long ADPRibose chains linked to the PARP enzyme would be responsible for the dissociation of the chromatin structure, and would then be degraded by the de-ADPRibosylating enzyme to allow reassociation of histones to DNA.

Boulikas (1993b) also proposed a model showing how pADPR chains, linked to the automodified form of PARP might be involved in removing histones from matrix associated regions of chromatin (MARs). Indeed, poly(ADPRibosyl)ated histone H1 might contribute to the destabilization of nucleosome structure, unfolding the DNA around the core histone octamer. The complete removal of histones from short sequences of DNA (1-4 nucleosomes) seems to be required for repair as well as for initiation of DNA replication and transcription (Boulikas, 1993b)

In rat testis, a functional form of PARP has been identified which did not seem to be an intrinsic component of the nuclear matrix; this form of PARP was rather

indirectly associated to the matrix structure (Quesada et al., 1994).

It has been shown that the modification of the linker histone H1 by pADPR alters drastically chromatin conformation (de Murcia et al., 1988; Boulikas, 1990). It has been demonstrated that the stage- and testis-specific histone H1 variant H1t are the preferential ADP-ribose acceptors among acid-soluble chromosomal proteins in rat testis (Quesada et al., 1990).

Poly(ADPR)polymerase has been found preferentially associated to transcriptionally active chromatin domains (Hough and Smulson, 1984). The microheterogeneity of H1 is known to play a role in the compaction of DNA into the nucleosome fiber. Among the H1 variants, H1t exerts the lowest condensing effect (De Lucia et al., 1994) and is mostly associated with transcriptionally active chromatin regions (De Lucia et al., 1996). Moreover, the same variants appeared to be ADP-ribosylated to different extents. Thus, taken together these findings indicate that poly(ADP-ribosylation) of histone proteins could contribute to the structural dynamics characteristic of the transcriptionally competent chromatin.

Our studies have explored the possible role of ADP-ribosylation reactions during spermatogenesis; the spermatogenesis offers a good model to investigate the relationship between poly(ADP-ribosylation) and structural and functional changes that chromatin undergoes during cellular differentiation.

**Figure 2.** Temporal appearance of germinal cells during development of rat seminiferous epithelium.

Data are expressed as a percentage of total cells, solubilized by collagenase digestion from seminiferous tubules, characterized as haploid spermatids, diploid and tetraploid spermatocytes, on the basis of their DNA content determined by cytofluorimetric analysis.

### 3. Protein level

In all cellular events involving poly(ADP-ribosylation), the state of chromatin represents a signal. It has been shown that poly(ADP-ribosylation) could affect chromatin structure by direct covalent modification of chromosomal proteins and by non-covalent interaction with histones (Lautier et al., 1993; Panzeter et al., 1992) due to the high number of long and branched pADPR chains linked to the PARP itself. A modulation in chromatin superstructure, induced by synthesis and degradation of pADPR, has been visualized by de Murcia et al. (1988) using electron microscopy.

Moreover, several nuclear proteins have been identified as ADP-ribose acceptors *in vivo* in different systems (Althaus and Richter, 1987): the list includes both structural chromosomal proteins (histones, HMGs, nuclear matrix proteins, etc.) and nuclear enzymes (DNA polymerase  $\alpha$ , DNA ligase, Topoisomerase I and II, etc.).

## II. Results

The temporal appearance of germinal cells in the rat seminiferous epithelium has been confirmed by cytofluorimetric analysis of total germinal cells isolated by collagenase digestion from testes of rats of different age. **Figure 2** shows that the seminiferous epithelium contains different numbers of three cell types, a function of animal age. On the basis of their DNA content rat germinal cells can be discerned as haploid cells (spermatids), diploid cells (mainly secondary spermatocytes), and tetraploid cells (primary spermatocytes). These three cellular populations are indicative of different stages of germinal cell differentiation. Indeed, the pachytene stage occurs in the interval between 25-35 days of age which is the longest portion of the prophase, characterized by the formation of the synaptonemal complexes and by a high level of genetic recombination. A difference is evident in the percentages of diploid/haploid cells in an interval from 28-32 days of postnatal age; the amount of tetraploid cells is always less than 25% and does not change significantly until 60 days of age.

**Figure 3** shows that PARP activity varies in rat testis in animals of different ages; the major level of PARP activity was detected in testes of 30 day-old animals coincident with a crucial phase of the spermatogenesis (meiosis), when the germ cell content (diploid/haploid ratio) changes drastically in the seminiferous tubules.

Moreover, the PARP enzyme is specifically present in the seminiferous epithelium and its activity can be

**Figure 3.** Poly(ADPR)polymerase activity in testis of rats of different ages.

The specific activity values of the enzyme are reported, as determined in isolated nuclei, with the enzymatic assays described in Materials and Methods.

Each value represents the average of four experiments done in duplicate. **S.T.:** seminiferous tubules isolated by collagenase digestion. **Crypt.:** artificial cryptorchid testes obtained by elevating the temperature to 37°C and placing them in the body cavity.

**Figure 4:** Levels of PARP mRNA in testis and prostate of differently aged rats.

Northern-blot analysis of total RNA extracted from testis and prostate of 30-days and 60-days old rats. Aliquots of 20 mg (A) and 50 mg (B) of RNA were hybridized with [<sup>32</sup>P]-labelled pRat cDNA probe. The migration of 3,6 Kb of PARP mRNA is reported on the right, and that of 28S and 18S ribosomal RNA on the left.

**Figure 5.** Cytofluorimetric analysis of isolated rat germ cell populations.

Fractions of rat germ cells isolated by centrifugal elutriation, were analyzed after propidium staining, by cytofluorimetry. (A) total germinal cells isolated by collagenase digestion from testes of 45 days old rats; (B) Fraction IV containing haploid spermatids; (C) Fraction VI containing diploid secondary spermatocytes; (D) Fraction VII containing tetraploid primary spermatocytes.

functionally related to the ongoing process of spermatogenesis, since it is drastically reduced in testes in which artificial cryptorchidism is induced.

Preliminary results obtained by Northern blot analysis of total RNA extracted from testes of rats of different ages showed that the expression level of PARP varies specifically in testis (not in other tissues) in relation with the animal age (**Figure 4**). The higher PARP expression level was observed in testes from 60-day old rats and could be correlated to the post-meiotic gene expression observed in mammals for instance to that for oncogenes (Erikson, 1990).

Using the procedure illustrated on **Table 1** the three cellular fractions have been isolated by centrifugal elutriation. Round spermatids were identified by optic microscope mainly in fractions V, whereas fraction VI contains mainly secondary spermatocytes, and fraction VII primary spermatocytes (data not shown). The enrichment rate obtained in these fractions was determined by cytofluorimetric analysis. **Figure 5** shows that on fraction IV, VI and VII approx. 85% enrichment was obtained for haploid, diploid and tetraploid cells respectively. Among these fractions, fraction VII represents a particularly interesting sample since it contains the highest percentage of tetraploid spermatocytes, the cells implicated in the pachytene phase of the meiotic division characterized by a high rate of RNA synthesis and the expression of stage-specific chromosomal proteins (histones TH2B and H1t).

**Figure 6:** Uridine and Thymidine uptake in rat germ cell populations.

The values indicate the [<sup>3</sup>H] incorporation observed in the acid-insoluble material of isolated germ cell fractions.

**Figure 7.** Poly(ADPR)polymerase and poly(ADPR)glycohydrolase activity in rat germ cell populations.

The specific activity values of the two enzymes are reported, as determined in total cellular fractions, with the enzymatic assays described in Materials and Methods. Each value represents the average of four determinations done in duplicate.

Germinal cell viability was assessed measuring [<sup>3</sup>H]thymidine and [<sup>3</sup>H]uridine uptake. **Figure 6** shows that the isolated cellular populations are able to incorporate the two precursors into the TCA precipitable material, and that the highest incorporation value is associated with primary spermatocytes.

The three germinal cell populations were analyzed for poly(ADPR)polymerase and poly(ADPR)glycohydrolase content. The results of the enzymatic activity assays are reported on **Figure 7**. A different value of PARP specific activity was detected; the sample enriched on tetraploid spermatocytes shows an enzymatic activity of 1.5 mU/mg of DNA higher than the 0.88 mU/mg of DNA detected in diploid cells, and three times as much as the 0.48 mU/mg of DNA of haploid spermatids. On the contrary, the PARP specific activity did not change significantly (0.8-1.0 mU/mg of DNA) in the same three samples.

These findings have been confirmed by Western-blot analysis of the same samples, using polyclonal antibodies

directed against PARP. **Figure 8** shows that, using immunodetection, the amount of PARP in primary spermatocytes cellular extract is higher than that in secondary spermatocytes and haploid spermatids.

These results are in agreement with a previous report by Corominas and Mezquita (1985) showing different pADPR levels in successive stages of rooster spermatogenesis, and support the functional relationship between spermatogenesis and poly(ADPribosyl)ation we have postulated (Quesada et al., 1990).

A potential role for PARP in cellular differentiation has been inferred from studies on enzymatic activity and protein amounts during differentiation in rat astrocytes (Chambert et al., 1992) and 3T3LI preadipocytes (Smulson et al., 1995). Changes in PARP expression accompany also the differentiation of HL60 cells induced by retinoic acid (Bhatia et al., 1990) and the proliferation of lymphocytes with PHA (McKerney et al., 1989). Wein et al. (1993) reported a cell cycle-related expression of PARP in proliferating rat thymocytes. In this case, a translational regulation of PARP occurs in the G1 phase, whereas a translational as well as transcriptional activation of PARP occurs in the S phase of the cell cycle.

It seems that different mechanisms of regulation of PARP also accompany germinal cell differentiation. Variations in the enzymatic activity have been observed which can be interpreted as changes in the number of PARP molecules, their enzymatic stimulation, modulation of PARP synthesis at the transcriptional and post-transcriptional levels, as well as in terms of differences in pADPR turnover rates.

Our evidence indicates that an active poly(ADPribosyl)ation system is associated mainly with tetraploid spermatocytes raising the possibility that PARP

**Figure 8:** Poly(ADPR)polymerase levels in rat germ cells.

Western-blot analysis of PARP levels in crude extracts of (1) haploid spermatids, (2) secondary spermatocytes and (3) primary spermatocytes. (A) Coomassie-Blue staining; (B) immunodetection with polyclonal antibody directed against human PARP.

in agreement with findings already reported in other systems.

We have also examined the level of ADPribosylation in the same fractions, taking into account both the extent of the modification and the size of the ADPribose oligomers. **Figure 10** shows DNA, proteins, PARP and pADPR content of the three fractions. In particular, the percentage of pADPR which was found associated with nuclear matrices prepared from nuclei incubated with 200  $\mu$ M [ $^{32}$ P]NAD is approximately 10% of the total nuclear

**Figure 9:** Isolation procedure of rat testis nuclear matrix.

The distribution of DNA, proteins and newly synthesized  $^{32}$ P-RNA in different chromatin fractions is also reported.

enzyme acts as a modulator of the nuclear processes that occur in these cells by its automodification or heteromodification (via covalent bond or non-covalent interaction) of specific nuclear proteins.

As in other experimental systems we can presume that poly(ADPribosyl)ation acts by inducing structural changes of chromatin, thereby facilitating DNA metabolism, which involves various protein-protein and DNA-protein interactions. The high rate of replicational activity of spermatocytes is well known and is accompanied by DNA recombination, a finely controlled crucial event during spermatogenesis ensuring integrity of the genetic material.

In order to determine the distribution and activities of the poly(ADPribosyl)ation molecules inside the nucleus, we have isolated and characterized different chromatin fractions, according to the procedure illustrated in **Figure 9**. Moreover, these fractions have been analyzed for their ability to incorporate [ $^{32}$ P]CTP in newly synthesized RNA. A high level of transcriptional activity is present in the DNase I-sensitive chromatin and also in the nuclear matrix

**Figure 10:** DNA, Proteins, PARP and pADPR content (%) of different chromatin fractions isolated after incubation of intact nuclei with  $^{32}$ P NAD.

1. DNase I-sensitive chromatin; 2. DNase I-resistant/ 2M NaCl extractable chromatin; 3. Nuclear matrix.

activity. It is noticeable that the pADPR content was not directly related to the amount of both DNA or protein. These results seem to indicate that the nuclear matrix is enriched in ADPribosylated proteins.

To characterize the kind of pADPR synthesized in nuclei, aliquots of nuclear fractions were subjected to alkaline hydrolysis (pH 12), thus detaching the intact polymer, which was then processed for electrophoresis on sequencing polyacrylamide gels and autoradiography. The typical ladder of pADPR indicates that a polymer population is synthesized in isolated nuclear matrix different from that synthesized in DNase I-sensitive and -resistant chromatin fractions; the matrix polymer is enriched in ADPR chains longer than 20 moieties, and in a fraction to the top of the gel which is known to be

enriched in branched polymers (**Figure 11**). Thus, nuclear proteins appear to be modified by three classes of pADPR differing in length and complexity.

To identify ADPribose acceptors, protein components extracted by 2M NaCl from nuclei were separated by means of 20% polyacrylamide gel electrophoresis on acetic-acid-urea (1st dimension) and SDS 15% polyacrylamide gel electrophoresis (2nd dimension) and processed for autoradiographic analysis. Autoradiography of [<sup>32</sup>P]ADP-ribosylated nuclear proteins revealed that

**Figure 11:** Autoradiography of <sup>32</sup>P-pADPR analyzed on a 20% polyacrylamide sequencing slab-gel.

1. Nuclear matrix; 2. DNase I sensitive chromatin; 3. DNase I resistant/ 2M NaCl extractable chromatin.

**Figure 12:** Electrophoretic pattern of loosely-bound chromosomal proteins.

2D-gel analysis (see Materials and Methods) of 2M NaCl extractable chromosomal proteins isolated from intact nuclei incubated with <sup>32</sup>P NAD.

radioactivity was associated to histone-like proteins and to component(s) with low electrophoretic mobility and high molecular weight which can be identified as the automodified form(s) of PARP.

Our results are in line with recent reports on the presence, within the nucleus, of multiple classes of PARP molecules, representing different functional forms of the enzyme. Others have reported the presence of variable amounts of PARP in nuclear matrices isolated from different sources (Wesierska-Gadek and Sauerman, 1985; Cardenas-Corona et al., 1987; Alvarez-Gonzales and Ringer, 1988). According to Kaufmann et al. (1991) the recovery of PARP in rat liver nuclear matrix varies with the extraction conditions, since the association of the enzyme to this structure is mediated by the formation of intermolecular disulfide bonds. Thus, the amount of pADPR (10%) associated with the rat testis nuclear matrix is far from being negligible.

An interesting hypothesis can be drawn concerning the well known automodification mechanism of PARP: the presence of a portion of ADPR tightly associated to the nuclear matrix raises the possibility of the occurrence of an auto-ADPRibosylated form of the enzyme anchored to the nuclear matrix.

In order to identify the ADPRibose acceptors among the rat testis nuclear proteins their ADPRibosylation has been induced in intact nuclei, by incubation with  $^{32}\text{P}$ -NAD. Acid-soluble proteins have been then extracted with 0.2 M  $\text{H}_2\text{SO}_4$  and separated by reverse-phase HPLC as reported in Materials and Methods. In a one step procedure, several histone and non-histone proteins have been purified, which appeared to be ADPRibosylated in different extent. The histogram in **Figure 12** shows the specific radioactivity calculated for each of the purified acid-soluble proteins. This analysis indicates that it is histone H1 which is mainly ADPRibosylated, compared to core histones and HMG like chromosomal proteins. Moreover, among the different histone H1 variants, H1t represents a better ADPRibose acceptor.

**Figures 13 and 14** show the reverse phase (RP)-HPLC pattern of H1 variants. Indeed, the autoradiography of purified proteins determined by 15% polyacrylamide gel electrophoresis in the presence of SDS, confirmed the preferential ADPRibosylation of the tissue- and stage-specific variant of the histone H1, H1t. Moreover, the H1t histone H1 variant appeared to be modified by ADPRibose oligomers ranging from 4 to 20 residues compared with no longer than 12 ADPR residues on H1a (data not shown).

The modification of the linker histone H1 by ADPRibosylation is well documented in different systems. The ADPRibosylation sites have been also identified as the  $\gamma\text{COOH}$  group of the two glutamic residues in the N-terminal and C-terminal domain of the protein, besides the COOH-terminal group itself. All these residues are conserved in the histone H1 variants and this means that

the different extents of their ADPRibosylation can not be explained on the account of unavailability of ADPRibosylation sites.

The existence of a non-covalent interaction of pADPR molecules with histone proteins has been described. This represents a different ADPRibosylation mechanism, due to

**Figure 13:** Purification procedure of  $^{32}\text{P}$  ADPRibosylated nuclear proteins.

The histogram reports the specific radioactivity value that has been calculated for single component purified by reverse-phase HPLC.

the long and branched polymers linked to the PARP, able to compete with DNA for histone binding (Panzeter et al., 1992). Both the covalent and non-covalent ADPRibosylation of histone H1 variants has to be taken into account to understand how PARP molecules modulate chromatin structure.

The differential activation of gene expression between hetero- and euchromatin is sustained by a non-uniform

distribution of somatic and tissue-specific H1 variants. However, the specific involvement of different H1 variants in transcriptional processes needs to be further investigated; in this scenario, the ADPribosylation could play an important role.

### III. Conclusions

Our data have some important implications with regard to the role and versatility of the poly(ADPribosyl)ation reactions in mammalian spermatogenesis.

(i) Regulation of the poly(ADPribose) turnover, variations in the number of PARP molecules synthesized, as well as changes in PARP transcription level, all seem to accompany the earlier phases of the germinal cell differentiation.

(ii) Three classes of poly(ADPribose) molecules of different length and complexity modify chromosomal proteins and PARP in the three chromatin fractions which were isolated (DNase I-sensitive DNase I-resistant chromatin, and nuclear matrix) is implicated in DNA replication, repair and transcription.

(iii). The testis-specific histone H1t is the main poly(ADPribose) acceptor, together with PARP itself, in rat germ-cells. Short oligomers modify covalently histone components whereas long and branched polymers are bound to non-histone proteins (PARP, nuclear matrix proteins, etc.).

Two general conclusions can be drawn: (i) The observation of a poly(ADPribosyl)ation system

particularly active in pachytene spermatocytes is in agreement with the proposed role of PARP as a guardian of the genome in preventing aberrant recombination. Such a role is particularly important in primary spermatocytes undergoing the pachytene phase of the meiotic division, characterized by DNA recombination events.

(ii) The observation of the preferential ADP-ribosylation of the histone H1 variant H1t, among the chromosomal proteins, can be related to the different roles attributed to the H1 variants in the compaction of DNA into the nucleosome fiber. Among the H1 variants H1t exerts the lower condensing effect and is mostly associated with transcriptionally active chromatin regions. This can explain why H1t is specifically expressed in pachytene spermatocytes. The different ADPribosylation patterns of histone H1 variants can amplify their role as modulators of chromatin structure, in relation to the various nuclear events that require chromatin remodelling.

#### **How to study the molecular and cellular events of the spermatogenesis using poly(ADPribosylation) as a marker.**

As already stated, to understand the etiology of chromosomally abnormal gametes, the knowledge of the normal mechanisms of chromosome behaviour is required.

To obtain this goal it would be advantageous to understand all the events of meiosis carried out by purified spermatocytes in culture. Procedures for separation of

**Figure 14:** Reverse-Phase HPLC of <sup>32</sup>P ADPribosylated histone H1 variants.

Chromatographic pattern of histone H1 variants separated by reverse-phase HPLC on a Vydac C4 silica column (see Materials and Methods).

The Figure shows also the electrophoretic pattern on SDS 15% polyacrylamide gel and the corresponding autoradiography of proteins contained in each peak.

germ-cells, such as the centrifugal elutriation, allow the isolation of cell populations at defined stages of differentiation. This method provides large amounts of meiotic cells and round spermatids suitable for biochemical studies (Quesada et al., 1996). The purity of the fractions is about 90% with a cell survival of 99%. Thus gene expression can be analyzed during the different stages of spermatogenesis with this approach.

Recently, short-term cultures of pachytene spermatocytes have been assessed that will be useful for investigating the defining events of pachytene substage of meiotic prophase, namely maintenance of chromosome pairing and recombination (Handel et al., 1995).

Rats of different ages can also be used to study the progression of germinal cell differentiation; since the spermatogenesis is a synchronous process, it is possible to correlate the presence in the seminiferous epithelium of different amount of each kind of germinal cells to the animal age.

Synaptonemal complex analysis is a novel approach for identifying substances hazardous to the germ cells. This sensitive cytological procedure reveals induction of structural damages and pairing abnormalities in SCs of meiotic prophase chromosomes, together with other germ-line toxic effects, and can allow an estimate of chemical mutagenicity (Allen et al., 1987).

To study changes in nuclear and chromatin composition and genomic activity during spermatogenesis several agents can be used to alter sex-organ development.

Cryptorchidism can be artificially-induced in animals maintained on a retinol-depleted diet since vitamin A has long been known to be essential for normal male reproductive function (La Borde et al., 1995). Spermatogenesis can be restored by the oral administration or intratesticular injection of retinoids which have been demonstrated to activate cell division in type A spermatogonia and induce their differentiation *in vitro*.

Cis-DDP is a drug frequently used in the treatment of testicular neoplasia; Cis-DDP determines changes in Sertoli cells function and is responsible for spermatid killing (Chu, 1994). Cis-DDP can be administered acutely or chronically in combination with a gonadotrophin releasing hormone analogue to evaluate the effects of this agent on spermatogenesis, with the aim to study the damage induced by chemotherapeutic drugs, and whether pretreatment with LRA is able to prevent this damage (Scott et al., 1996).

Undifferentiated germ cells can offer a rapid, sensitive and inexpensive alternative to screening for teratogenicity and genotoxicity of diverse chemicals on live animals. As an example methyl-methanesulphate (MMS) can be used which is known to affect mouse sperm chromatin structure and testicular cell kinetics. MMS is a potent electrophilic,

monofunctional alkylating agent which induces clastogenic damage including dominant lethal mutations. It has been demonstrated that MMS alkylates cysteine -SH groups in sperm protamines, thereby destabilizing sperm chromatin structure and leading to chromosomal breakage and mutations (Evenson et al., 1993).

Alternatively, physical agents can be used to alter cell progression. The DNA damage induced after exposure to ionizing radiation can be measured at different cellular stages of spermatogenesis as well as the DNA repair rate, as well as in relation to the apoptotic index (Van Loom et al., 1991).

The comet assay represents a sensitive a rapid method for DNA damage detection on individual cells, able to size highly fragmented DNA and DNA repair efficiency (Fairbairn et al., 1995)

Treatment with okadaic acid (OA) seemingly abrogates a checkpoint that holds mid-pachytene spermatocytes in prophase for several days. Since OA is a polyether monocarboxylic acid able to inhibit several protein phosphatases, the phosphorylation state of critical proteins may be an important element of spermatogenic meiosis cell-cycle control (Wiltshire et al., 1995).

Going from genes to their product, the cellular protein pattern is also representative of the functional state of the cell. Moreover post-translational modification of proteins represents also a signal of the normal or abnormal progression of the cell cycle. Several post-translational protein modifications can be directly related to the nuclear metabolism.

The role of protein modification, could be studied *in vitro* and/or *in vivo*. Reconstitution experiments, involving addition of specific proteins to DNA *in vitro* could help determine how these proteins affect chromatin structure. A direct but very difficult method would be to specifically prevent their synthesis *in vivo* and then determine the resulting modification on chromatin structure.

Two dimensional gel electrophoresis of proteins can be used to detect and purify hundreds of proteins from a single sample simultaneously. The protein map of rat spermatocytes and round spermatids has been obtained with this method, and several proteins were successfully identified in the SWISS-PROT protein database, in order to highlight changes in protein expression during meiosis (Cossio et al., 1995).

On the basis of these considerations and in the light of the experimental evidence already available, it is now possible to better understand the functional correlation between poly(ADPribose)ylation and spermatogenesis. Alterations in the poly(ADPribose)ylation system might be useful markers in gene therapy of germ-cell line.

#### IV. Materials and methods

**Materials.** Wistar rats (28-130 days of age) were used in all the experiments.

[U<sup>14</sup>C]NAD<sup>+</sup>, nicotinamide [U<sup>14</sup>C]adenine dinucleotide ammonium salt (248 mCi/mmol), [<sup>32</sup>P]NAD<sup>+</sup>, nicotinamide adenine dinucleotide di(triethylammonium) salt (adenylate <sup>32</sup>P), 1000 Ci/mmol, [<sup>32</sup>P]CTP (3000 Ci/mmol), [<sup>3</sup>H] methylthymidine (40 Ci/mmol) and [<sup>3</sup>H]Uridine (25-30 Ci/mmol) were supplied by Amersham International plc. DNase I (EC 3.1.21.1) (2,000 U/mg), collagenase type XI, phenyl-methyl-sulphonyl-fluoride (PMSF), leupeptin, spermine, and spermidine were obtained from SIGMA Chemical Company; guanidine thiocyanate was from FLUKA, pure grade. Electrophoretic molecular weight markers were purchased from Pharmacia, X-Omat RP films from Kodak, and nitro-cellulose filters (0.45 μm pore size, type HA) from Millipore. Reverse-Phase HPLC silica 300-C4 columns were from Vydac (ODS 5 mm particles, 0.5 x 25 cm)

**Isolation of germinal cells by centrifugal elutriation.** Testes from two rats were decapsulated, resuspended in 10 ml of Dulbecco's minimal essential medium (DMEM) and seminiferous tubules free of the interstitial tissues were obtained by collagenase treatment (0.25 mg/ml). The seminiferous tubules were then incubated at 30°C for 60 min in DMEM containing 0.25 mg/ml collagenase, 0.075 mg/ml DNase I, and 0.5% bovine serum albumin (BSA). After incubation the cell suspension was centrifuged for 10 min at 1,200g. Aliquots of the pellet were complexed with propidium and subjected to cytofluorimetric analysis in a Becton-Dickinson cytofluorimeter. Total germinal cells resuspended in DMEM, in the presence of 0.1 mg/ml DNase I and 0.5% BSA, were separated into fractions enriched in various cell types by sedimentation at unit gravity (centrifugal elutriation), as described by (Quesada et al., 1996).

A cell suspension of 10 ml (180-220x10<sup>6</sup> cells obtained from two testes of 40-45 day aged rats) was loaded into a JE-6 Beckman elutriator rotor and separation was performed with speeds of 3,000-2,000 rpm and flow rates of 13-40 ml/min (Table 1). The buffer employed was phosphate buffered saline (PBS) containing 0.5% BSA; several fractions of 50 ml were collected. Aliquots of the pellet of single fractions were complexed with propidium and subjected to cytofluorimetric analysis in a Becton-Dickinson cytofluorimeter.

Isolation of sub-cellular fractions was carried out essentially as described by (Quesada et al., 1996). Testes were homogenized in a medium containing 0.32 M sucrose, 2 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol, centrifuged at 2,000g for 10 min and the nuclei recovered in the sediment. The supernatant was centrifuged at 20,000 g for 60 min in a SW40 rotor, to obtain in the pellet the microsomal fraction separate from the soluble fraction.

**[<sup>3</sup>H] Thymidine and [<sup>3</sup>H] Uridine incorporation.** Seminiferous tubules were labelled for 1 hr at 37°C in DMEM in the presence of 30 mCi/ml of [<sup>3</sup>H] methylthymidine or [<sup>3</sup>H]Uridine. The tissue was washed three times with the medium and the cells isolated as described above. The cellular fractions were washed with 5% trichloroacetic acid, ethanol, ethanol/ether and ether to remove unbound radioactivity. The residue was suspended in 0.2 M HClO<sub>4</sub> and portions of the solution were used for the determination of radioactivity on a Beckman LS8100 liquid scintillation spectrometer.

**[<sup>32</sup>P] RNA synthesis.** RNA synthesis was followed by incubation of nuclei in the presence of [<sup>32</sup>P]CTP according to (Greengard and Zif, 1984) and [<sup>32</sup>P]RNA was measured as 25% trichloroacetic acid-insoluble radioactivity.

**Isolation of chromatin fractions.** Rat testis nuclei were isolated by homogenization and differential centrifugation, as previously described (Quesada et al., 1990). Proteases were irreversibly inhibited by 1 mM PMSF and 0.1 mM leupeptin. The nuclear matrix isolation procedure was essentially as described by (Tubo and Berezney, 1987) and consisted in the endogenous digestion of isolated nuclei 45 mins at 37°C, followed by a three times repeated extraction with a high salt buffer (2 M NaCl, 0.2 mM MgCl<sub>2</sub>, 1 mM PMSF in 10 mM Tris-HCl pH 7.4) followed by three washes with low-salt buffer (0.2 mM MgCl<sub>2</sub>, 1 mM PMSF in 10 mM Tris-HCl pH 7.4). The nuclear matrix, was then re-suspended in one third volume of low-salt buffer.

Alternatively, nuclei were suspended in 10 mM TRIS-HCl pH 7.5, 1 mM EDTA, 1 mM PMSF, digested with DNase I (600U/mg of DNA) and lysed for 1 hr at 0°C. Lysed nuclei were centrifuged for 20 min at 12,000 g, the supernatant collected and the pellet re-extracted twice as above (DNase I resistant chromatin). The insoluble fraction was digested with DNase I (1,000 U/mg DNA) in 60 mM TRIS-HCl pH 7.5, 60 mM NaCl, 20 mM MgCl<sub>2</sub> at 37°C for 1 hr, to obtain after centrifugation 12,000g 20 min, the nuclear scaffold.

**Nuclear protein extraction.** Acid-soluble proteins contained in nuclei, and nuclear fractions were extracted three times by 1 h stirring at 4°C in 0.2 M H<sub>2</sub>SO<sub>4</sub>, and centrifuged at 10,000g for 15 min. The extracts were pooled and proteins precipitated with 6 vols. of ice-cold acetone at -20°C overnight. Precipitates were collected by centrifugation at 18,000g for 20 min at -10°C

**Poly(ADPribose)polymerase and poly(ADPR)-glycohydrolase activity assay.** In a typical PARP activity assay, the reaction mixture (final volume 250 μl) contained: 100 mM Tris-HCl pH 8, 14 mM β-mercaptoethanol, 10 mM MgCl<sub>2</sub>, 4 mM NaF, 200 μM [<sup>14</sup>C]NAD (10,000 cpm/nmol), 12 μg DNase I, and, as enzyme source, an amount of cells or sub-cellular fractions corresponding to 30 μg of proteins. After 10 min incubation at 20°C, the reaction was stopped with ice-cold trichloroacetic acid and the radioactivity present in the acid-insoluble material, collected on a HAWP (0.45μm) filter, determined on a Beckman LS8100 liquid scintillation spectrometer. One enzymatic unit was defined as the enzyme activity catalyzing the incorporation, per minute at 20°C, of one μmole of ADPribose into acid-insoluble material.

The PARG activity assay was performed in a standard reaction mixture containing 50 mM potassium phosphate (pH 7.2), 10 mM β-mercaptoethanol, 100 μg/ml BSA, 10 mM [<sup>14</sup>C] poly(ADPribose) (20 residues long on average) and, as enzyme source an aliquot of cells or sub-cellular fractions corresponding to 30 μg of proteins in a total volume of 100 μl. After 10 minutes of incubation at 37°C, the reaction was stopped with ice-cold 20% trichloroacetic acid and the radioactivity present in the acid-soluble material determined on a Beckman LS8100 liquid scintillation spectrometer. One enzymatic unit was defined as that liberating 1 μmole of ADPribose, per minute at 37°C.

**Blotting experiments.** Activity-blots and immuno-blots were performed as described by (Simonin et al., 1991). Aliquots of 10<sup>7</sup> cells were suspended in 100 μl of 50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA, 1 mM PMSF and sonicated with 60-s pulses at 180V. The crude extract, after incubation at 45°C for 15 min in 50 μl of 50 mM Tris-HCl pH 6.8, 6 M urea, 6% β-mercaptoethanol, 3% SDS, was separated on 10% polyacrylamide slab-gel in the presence of 1% SDS and

electrotransferred onto nitro-cellulose sheets at 4°C for 2 hrs at 200 mA. For activity-blot experiments proteins transferred onto nitrocellulose sheets were incubated for 1 hr at room temperature in renaturation buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM DTT, 0.3% (v/v) Tween 20, 20  $\mu$ M Zn(II)acetate, 2 mM MgCl<sub>2</sub>) containing DNase I activated DNA (2  $\mu$ g/ml) and, for 2 hr more, in the same buffer containing [<sup>32</sup>P]NAD 1  $\mu$ Ci/ml. The blots were then washed several times with the renaturation buffer, dried and analyzed by autoradiography. For immuno-blot experiments nitrocellulose sheets were treated for 1 hr with the blocking solution (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% (v/v) Tween 20 and 3% (w/v) gelatin). Incubation with anti-human PARP antibodies was performed, for 2 hrs at room temperature in the same solution supplemented with 0.3% gelatin. The blots were then washed twice with TBS-Tween and antibody binding was detected by using alkaline phosphatase conjugated goat anti-rabbit IgG second antibody with BCIP/NBT (SIGMA) tablets as substrate for the color reaction.

For Northern blot experiments total cellular RNA (20-50  $\mu$ g per sample) was first separated by electrophoresis on a 1% agarose gel containing 6% formaldehyde, then blotted onto a Hybond N membrane and hybridized with a fragment of rat PARP cDNA (pRatC) [<sup>32</sup>P]-labelled using a Multiprime DNA labelling kit (Amersham International plc).

**Protein and DNA assay.** Protein concentration was determined following the method described by (Burton, 1968) using bovine serum albumin as standard. DNA content was determined on the basis of the absorbance at 260 nm (1.0 OD<sub>260nm</sub> = 50  $\mu$ g /ml DNA) or by the diphenylamine method described by Burton (1968).

**Electrophoretic analysis.** Acid-soluble nuclear proteins were analysed by electrophoresis on urea-acetic acid 20% polyacrylamide slab-gels (pH 2.9) and, SDS 7-15% polyacrylamide slab-gels, as described by (Quesada et al., 1996). Each labelled sample was run in duplicate and either stained with Coomassie Brilliant Blue R-250 or autoradiographed.

**Analysis of reaction products.** Intact [<sup>32</sup>P]poly(ADP-ribose) moieties incorporated into the proteins were detached by incubation at 60°C for 3 h with 10 mM Tris, NaOH pH 12, 1 mM EDTA. Samples were extracted with phenol/CHCl<sub>3</sub>/isoamyl alcohol (49:49:2), dried in a Speed-Vac and dissolved in 50% urea, 25 mM NaCl and 4 mM EDTA, pH 7.5, to be analyzed on 20% polyacrylamide slab-gel (Panzeter and Althaus, 1990)

**Reverse-Phase HPLC analysis.** Acid soluble nuclear proteins were purified by reverse-phase HPLC using a 300-5 C4 silica column and a gradient elution system formed by 0.1% trifluoroacetic acid (solvent A) and 95% CH<sub>3</sub>CN in 0.1% trifluoroacetic acid (solvent B). The gradient elution was from 15 to 65% of solvent B in 70 mins (slope 0.65%/min).

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*Quesada: Poly (ADP-ribosyl)ation in mammalian spermatogenesis*

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Dear Piera, ciao!

Many thanks for this timely review. I have made a number of editing changes. Please check carefully and please send it back by express mail. Need to send the book before Dec 22.

I was unable to open the color figures of your diagrams. You may want to send me color prints.