The vector component (pMB9) is not shown. The enzymes used were: E, EcoRI; H, HindIII; Bg, BglII; X, XhoI; Sg, SaliI; Hp, HpaI; B, BamHI; Sp, SaliI; A, AspAI; K, KpnI; and S, Smal. The numbered subfragments above the map refer to additional pSZ clones.

Subfractionation of the endonuclease-HindIII-produced fragment containing the end of the T-DNA has proved difficult, as few restriction endonucleases cleave in this region. In addition to those enzymes shown in Fig. 1, endonucleases HgiAI and HaeIII all generate large (greater than 0.9 x 10^6 daltons) fragments in this region. We have been screening enzymes whose recognition site comprises four bases in order to break up this region into lengths suitable for sequencing.

One piece of gross sequence information obtained to date is that, at a distance corresponding to 3 x 10^6-4 x 10^6 daltons to the left of the T-DNA, is a short region of high GC content, as evidenced by retardation of restriction-endonuclease-produced fragments on acrylamide gels and relative buoyant density.


Characterization of poly(adenosine diphosphate ribosylated) protein isolated from mouse L1210 cells in vivo

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Poly(ADP-ribose) is the product of a chromatin-bound enzyme system that catalyses the addition of the ADP-ribose moiety of NAD⁺ to nuclear proteins, with the concomitant release of nicotinamide. ADP-ribosylation has been found to occur in the nuclei of all eukaryotic systems so far studied (Hilz & Stone, 1976; Hayashi & Ueda, 1977).

However, the role of ADP-ribosylation of chromosomal proteins in nuclear function is not clear. Most studies to date have been carried out in vitro, where the true characteristics of the ADP-ribose-protein complex may be altered. Analysis of proteins modified in vivo may therefore give a good indication of the function of this protein modification.

Mouse L1210 lymphoma cells at a density of 2 x 10^6/ml were incubated for 18 h with [2-3H]Adenosine to give 40 Ci/mmol of cells, which were then isolated and washed several times with high concentrations of ethanol to remove contaminating nucleotides. CsCl₂-density-gradient equilibrium centrifugation of this material under optimal conditions (a modification of the method

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of Rickwood et al. (1977)) gave excellent separation of ADP-ribosylated proteins from DNA and RNA. The material was precipitated by addition of trichloroacetic acid to a final concentration of 25% (w/v), left on ice for 4 h, then washed and centrifuged three times at 38000 g for 15 min. The resulting material was digested with the following: (a) snake-venom phosphodiesterase, EC 3.1.4.1; (b) snake venom phosphodiesterase plus alkaline phosphatase, EC 3.1.3.1; (c) spleen phosphodiesterase, EC 3.1.4.18; (d) ribonuclease A, EC 3.1.4.22; (e) deoxyribonuclease I; EC 3.1.4.5; (f) Pronase (Hilz et al., 1975); (g) base (Adamiit et al., 1978); (h) hydroxylamine (Adamiit et al., 1978). Increase in radioactivity in the supernatant of a 10 min, 10000 g centrifugation in 66% (v/v) ethan/0.5M-sodium acetate, pH 5.0, gave an indication of the amount of acid-insoluble material digested. Results of this preliminary characterization showed that 92% was solubilized with snake-venom phosphodiesterase, 17% with spleen phosphodiesterase, 14% with ribonuclease, 0% with deoxyribonuclease, 68% with Pronase, 87% with base, and 21% with hydroxylamine. This digested material was then subjected to a number of t.1.c. systems. Unambiguous linkage of ADP-ribose units to protein was found. Chain-length studies revealed that all polymerization occurs at the ADP-ribose acceptor site, where ADP-ribose polymerization is not coupled to protein phosphorylation. ADP-ribosylation of proteins by this enzyme is a complex process involving both protein-bound and free ADP-ribose. Further analysis of the transient products of these chemical digests showed that ADP-ribose was indeed produced.

Digestion of gradient material overnight with 0.2 M-NaOH will produce 5'-AMP from monomer, 3'-AMP from RNA, but will not digest polymer. The results of two different t.1.c. systems (Schwarz & Drach, 1975; E. Randerath & K. Randerath, 1965) indicated that, in fact, 15% of material was present as 3'-AMP, 55% as 5'-AMP and 20% remained on the origin as poly(ADP-ribose). Further digestion of the base-treated material with 3'-nucleotidase (EC 3.1.3.6) and 5'-nucleotidase (EC 3.1.3.5) followed by determination of percentage decrease in AMP and ADP-ribose showed that ADP-ribose was indeed produced. The above two studies gave an average chain length of polymer attached to protein of 1.2 ADP-ribose units. The linkage between polymer (ADP-ribose) and protein has been reported to be sensitive to both base and hydroxylamine (Nishizuka et al., 1969). T.l.c. analysis of the transient products of these chemical digests showed that ADP-ribose was indeed produced.

The biological function of this enzyme remains unknown. It has been postulated that the enzyme may be involved in DNA synthesis, transcription or repair (Hilz & Stone, 1976). During a recent study involving the screening of benzamide molecules as potentially specific inhibitors of the enzyme, it was found that exhaustively digest with snake-venom phosphodiesterase, deoxyribonuclease I and alkaline phosphatase, followed by a borate t.i.c. system capable of distinguishing adenosine from deoxyadenosine (K. Randerath & E. Randerath, 1965). This analysis showed the complete absence of deoxyadenosine and therefore of DNA. It was then possible to carry out further analysis of the properties of the system in vivo. It has been well established that a number of inhibitors exist capable of inhibiting poly(ADP-ribose) polymerase in vitro, including thymidine (Preiss et al., 1971). Thus, cells were taken 6 h before labelling and incubated with 5 mM-thymidine, 2 mM-3-aminobenzamide or 2 mM-3-methoxybenzamide, the latter two being inhibitors that have been developed in this laboratory and found to produce an even higher percentage inhibition than others previously described (M. R. Purnell & W. J. D. Whish, unpublished work). However, with these inhibitors only 10% inhibition of the ADP-ribosylation of protein was found. Chain-length studies revealed that although the radioactivities in AMP were the same, there was no detectable phosphoribosyl-AMP. This provides the interesting possibility that the inhibitors work at the polymerization step but not at the initiation step. It is therefore possible that more than one enzyme exists, one for initiation and one for polymerization.

It thus appears that the analysis of ADP-ribosylation in vivo can provide much insight into understanding the role of this complex system.

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The effect of phenones on poly(adenosine diphosphate ribose) synthetase from porcine thymus

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Poly(ADP-ribose) synthetase is a nuclear enzyme responsible for the covalent modification of proteins by ADP-ribose or an oligomer thereof. NAD is cleaved at the nicotinamide-ribose bond and the ADP-ribose is attached to either a protein or a protein-bound (ADP-ribose) chain, where n = 1-30.

The biological function of this enzyme remains unknown. It has been postulated that the enzyme may be involved in DNA synthesis, transcription or repair (Hilz & Stone, 1976). During a recent study involving the screening of benzamide molecules as potentially specific inhibitors of the enzyme, it was found that acetophenone and 3-aminacetophenone were inhibitory (M. R. Purnell & W. J. D. Whish, unpublished work). Nicotinamide is known to inhibit the enzyme, and the binding site is highly specific (Preiss et al., 1971). The effect of several phenones on the enzyme was examined to see whether the same specificity was required for inhibition by analogues of acetophenone.

Poly(ADP-ribose) synthetase was extracted from porcine thymus nuclei, isolated as described by Khan & Shali (1976), with 0.5 M-NaCl/100 mM-triethanolamine/HCl (pH 8.2)/10 mM-MgCl2/2.5 mM-dithiothreitol. The enzyme activity was determined by the incorporation of [3H]NAD+ into acid-insoluble material. The reaction mixture contained 100 mM-triethanolamine/HCl, pH 8.2, 10 mM-MgCl2, 2.5 mM-dithiothreitol, 50 μM [3H]NAD (sp. radioactivity 2 mCi/mmol) in 180 μl. The reaction was started by the addition of 20 μl of 0.5 M-NaCl extract.