Adenosine Diphosphate Ribosylation of Histone and Nonhistone Chromosomal Proteins with Oxidized Nicotinamide Adenine Dinucleotide and 2′-Deoxynicotinamide Adenine Dinucleotide Using Nuclei Isolated from Rat Liver and HeLa Cells†

Diane M. Lichtenwalner and Robert J. Suhadolnik*

ABSTRACT: By use of sodium dodecyl sulfate–polyacrylamide and acid–urea gel electrophoresis, evidence is presented that poly(adenosine diphosphate ribose) [poly(ADP-ribose)] polymerase of nuclei isolated from HeLa cells and rat liver preferentially transfers the ADP-ribose moiety from oxidized nicotinamide adenine dinucleotide (NAD^+) to the H1 histones. With nuclei isolated from normal rat liver, the minor H1 histone, H1°, is ADP ribosylated. With nuclei isolated from normal rat liver or HeLa cells, poly(ADP-ribose) polymerase preferentially transfers the 2′-deoxyadenosine diphosphate ribose (2′-dADP-ribose) moiety of 2′-deoxynicotinamide adenine dinucleotide ($2'dNAD^+$) to the nonhistone proteins. The average chain lengths of poly(ADP-ribose) from NAD^+ covalently bound to either HeLa or rat liver histone proteins are 5 and 7.2, respectively, whereas the average chain length of ADP-ribose associated with the nonhistone proteins of nuclei isolated from rat liver and HeLa cells is 1.7. With $2'dNAD^+$, the average chain length of the 2′dADP-ribose covalently bound to the histone and nonhistone proteins is 1.2. The difference in the average chain length and the marked differences in the ADP ribosylation of the histone and nonhistone proteins with NAD^+ and $2'dNAD^+$, respectively, demonstrate the importance of the 2′-hydroxyl group of NAD^+. The data presented here provide additional evidence to explain the earlier observations that the replacement of the 2′-hydroxyl group of NAD^+ with a hydrogen atom (1) causes a 10-fold increase in the inhibition of deoxyribonucleic acid (DNA) synthesis in nuclei isolated from normal rat liver and (2) restores the ability to inhibit DNA synthesis in nuclei isolated from neoplastic tissue and fetal rat liver [Suhadolnik et al., *J. Biol. Chem. 252*, 4134–4144 (1977)]. This inhibition of DNA synthesis in neoplastic tissue and fetal rat liver is ineffective with NAD^+, but is restored by $2'dNAD^+$.

Many eucaryotic nuclei possess an enzyme which polymerizes the ADP-ribose[1] moiety of NAD^+ into a polymer of repeating ADP-ribose units (Chambon et al., 1963; Nishizuka et al., 1967; Fujimura et al., 1967). Poly(ADP-ribose) ranges in size from monomers to polymers of ADP-ribose units (Tanaka et al., 1978). ADP ribosylation represents a new type of post-transcriptional change of proteins. In procaryotes, it mostly involves mono-ADP ribosylation, while in eucaryotes the homopolymer is formed (Hayaishi & Ueda, 1977). Poly(ADP-ribose) has been shown to be synthesized in vivo and in vitro (Ueda et al., 1975; Nishizuka et al., 1968; Otake et al., 1969; Ord & Stocken, 1977; Tanuma et al., 1977; Roberts et al., 1975) and covalently attached to histone proteins (Smulson & Roberts, 1973; Adamietz et al., 1976; Burzio et al., 1979), nuclear enzymes (Yoshihara et al., 1975; Müller & Zahn, 1976), and an endogenous poly(ADP-ribose) acceptor that is neither protein nor nucleic acid (Okazaki et al., 1976). The original proposal by Morton (1958) that NAD^+ may be important in the control of the growth of the cell has been well documented. Studies on mammalian cells have established that there is a correlation between the cellular concentration of NAD^+ and the growth of the cell (Winzerith et al., 1961; Ferris & Clark, 1971; Schwartz et al., 1974). The data obtained demonstrate that poly(ADP-ribose) could be involved in either DNA synthesis (Burzio & Koide, 1970, 1973), gene expression (Caplan & Rosenberg, 1975; Müller et al., 1974; Proctor & Casida, 1975), chromatin structure modification (Ueda et al., 1975; Giri et al., 1978), acidic protein modification (Colyer et al., 1973), cell proliferation (Colyer et al., 1973; Smulson et al., 1976; Kidwell & Burdette, 1974), or inhibition of RNA polymerase I and stimulation of RNA polymerase II (Müller & Zahn, 1976).

More recent findings indicate that ADP ribosylation may also be involved in the regulation of repressor activity. For example, Okayama & Hayaishi (1978) have demonstrated that nuclear protein A-24, which is composed of histone H2A and ubiquitin joined by an isopeptide, is ADP ribosylated in isolated rat liver nuclei.

In isolated rat liver nuclei, NAD^+ inhibits the incorporation of [^{3}H]dTTP into DNA (Yoshihara et al., 1975; Burzio & Koide, 1973). In isolated HeLa cell nuclei, NAD^+ enhances the template activity for DNA synthesis (Roberts et al., 1973; Smulson & Rideau, 1972). Replacement of the 2′-hydroxyl group of the adenosine ribose of NAD^+ with a hydrogen atom resulted in an NAD^+ analogue ($2'dNAD^+$) whose 2′dADP-ribose moiety is covalently bound to nuclear proteins (Suhadolnik et al., 1977). This transfer of 2′dADP-ribose to nuclear proteins results in the inhibition of incorporation of dTMP into DNA in isolated rat liver nuclei and HeLa cell nuclei. This communication examines the nature of the nuclear proteins which are modified by the posttranscriptional addition of ADP-ribose or 2′dADP-ribose by either NAD^+ or $2'dNAD^+$ in nuclei isolated from HeLa cells and rat liver.

Materials and Methods

Enzymes and Chemicals. Phosphodiesterase I (*Crotalus adamanteus* venom) (SVPD I, EC 3.1.4.1), bovine pancreas DNase I (EC 3.1.4.5), and bovine pancreas RNase (EC 2.7.7.16) were purchased from Worthington Biochemical

† From the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140. *Received January 18, 1979; revised manuscript received May 11, 1979.* This project was supported by a grant from the National Institutes of Health (GM-26134).

[1] Abbreviations used: ADP-ribose, adenosine diphosphate ribose; NAD^+, nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid.

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Corporation; SVPD I was further purified to remove 5′-nucleotidase activity (Keller, 1964). [*adenine*-2,8-^{3}H]Nicotinamide dinucleotide, [G-^{3}H]2′dATP, [α-^{32}P]ATP, and [α-^{32}P]2′dATP were purchased from New England Nuclear Corp. [α-^{32}P-AMP]NAD^+, [α-^{32}P-2′dAMP]2′$dNAD^+$, and [G-^{3}H]2′$dNAD^+$ were synthesized enzymatically, purified, and characterized as described previously (Suhadolnik et al., 1977).

Chromatography and Chemical Determinations. Descending paper chromatography was performed by using Whatman No. 3MM chromatography paper at 25 °C and 0.1 M phosphate buffer (pH 6.8)–$(NH_4)_2SO_4$–1-propanol (100:60:2, v/w/v) (Shima et al., 1969). Protein was measured by the procedure of Lowry et al. (1951).

Isolation of Nuclei. (*A*) *Rat Liver.* Male albino Sprague–Dawley rats (180–220 g) were fasted 12 h and decapitated. Nuclei were isolated (Blobel & Potter, 1966), washed twice in 0.25 M sucrose in TKM buffer (0.05 M Tris-HCl, pH 7.5 at 20 °C, 0.025 M KCl, 0.005 M $MgCl_2$), and centrifuged at 1500*g* for 5 min, 4 °C.

(*B*) *Hela Cells.* HeLa cells (ATCC No. CCL 2.2) were grown logarithmically in suspension culture in Earles MEM Spinner medium supplemented with 3.5% fetal calf serum and 3.5% calf serum. Cells were harvested by centrifugation at 800*g* for 5 min, 4 °C. Cells were washed in 10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 4 mM $MgCl_2$, and 6 mM 2-mercaptoethanol at 2×10^6 cells/mL. They were resuspended in the same buffer at 7×10^7 cells/mL (Hershey et al., 1973). After swelling in the buffer for 10 min at 4 °C, the cells were lysed by 10–15 strokes in a Dounce homogenizer (Kontes Glass Corp.). Nuclei were sedimented by centrifugation at 800*g* for 15 min at 4 °C.

Isolation of ADP-Ribosylated Nuclear Proteins. Nuclei (0.3 mg of protein) isolated from either rat liver or HeLa cells were incubated with 2 μCi of either [α-^{32}P-AMP]NAD^+ (10 Ci/mmol), [α-^{32}P-2′dAMP]2′$dNAD^+$ (10 Ci/mmol), or [*adenine*-2,8-^{3}H]nicotinamide dinucleotide (5 mCi/μmol) at 25 °C for 15 min with 80 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, and 250 mM sucrose in a total volume of 500 μL. After 15 min, the tubes were rapidly cooled on ice. Nuclear proteins were isolated according to the procedure of Marks et al. (1975). All operations were performed at 4 °C. Two milliliters of a solution containing 80 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 0.05 M $NaHSO_3$ (pH 7.2) was used to wash the nuclei three times. The pellet, obtained after centrifugation at 600*g* for 10 min, was washed three times with 0.15 M NaCl containing 1 mM PMSF and 0.05 M $NaHSO_3$. Histones were extracted from the pellet with 0.25 N HCl containing 1 mM PMSF and 0.05 M $NaHSO_3$. After removal of the 0.25 N HCl-soluble material (histones), the pellet containing the nonhistone proteins was suspended in 4 vol of deionized water (4 °C). Samples were dialyzed overnight against 15 vol of 50 mM Tris-HCl, pH 6.8, at 4 °C. To selectively extract H1 and H1° histones, an aliquot of the histone solution was made 5% with concentrated perchloric acid. The sample was chilled on ice for 30 min and centrifuged at 800*g* for 15 min, 4 °C. The pellet, containing the H1 histones, was taken up in 4 vol of deionized water, 4 °C, and dialyzed overnight against 15 vol of 50 mM Tris-HCl, pH 6.8, at 4 °C.

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. Samples prepared for $NaDodSO_4$–polyacrylamide slab gel electrophoresis contained 0.035 M Tris-HCl (pH 6.8), 0.7 M urea, 0.7% $NaDodSO_4$, 0.0014 M EDTA, 13.8% glycerol, 0.0015% 2-mercaptoethanol, and 0.0015% bromphenol blue. Samples were incubated at room temperature for ~16 h and

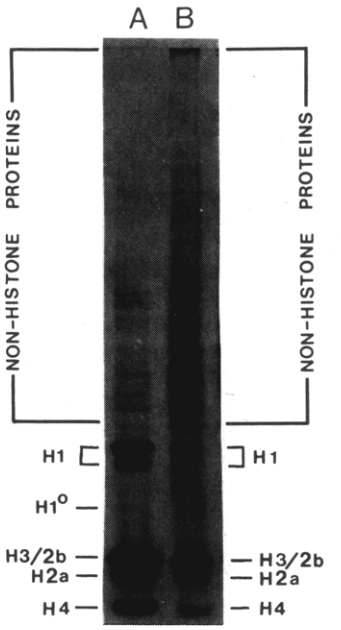

FIGURE 1: Photograph of representative rat liver (A) and HeLa cell (B) total nuclear proteins separated by the $NaDodSO_4$–polyacrylamide slab gel electrophoresis system detailed in Materials and Methods.

applied to the gels in the following amounts: total chromosomal proteins, 150 μg; nonhistone proteins, 100 μg; histone proteins, 50 μg; and H1 histones, 25 μg.

The polyacrylamide gel used for the $NaDodSO_4$–polyacrylamide slab gel electrophoresis contained 18% acrylamide according to Laemmli & Favre (1973), except that 0.75 M Tris-HCl was used in the separating gel (Thomas & Kornberg, 1975) and the ratio of acrylamide/*N*,*N*′-methylenebis-(acrylamide) was 30:0.15 (Bonner, 1975). The electrode buffer contained 0.05 M Tris, 0.38 M glycine, and 0.1% $NaDodSO_4$. A 1.0-cm stacking gel was prepared according to Laemmli & Favre (1973). Slab gels were 0.15 cm thick, 14.0 cm long, and 13.5 cm wide. Electrophoresis was performed at 30 mA until the tracking dye was 1.0 cm from the bottom of the gel. Gels were stained overnight at room temperature in 0.05% (w/v) Coomassie Brilliant Blue R in 1-propanol–acetic acid–water (25:10:65, v/v/v) (Fairbanks et al., 1971). Gels were destained electrically by using 7% acetic acid. A representative photograph of rat liver and HeLa cell total nuclear proteins separated by the $NaDodSO_4$–polyacrylamide slab gel electrophoresis system described above is shown in Figure 1. Gels were sliced into strips corresponding to each sample well and were scanned at 660 nm in a Gilford spectrophotometer (Model 2400) equipped with a gel scanning device. The radioactivity associated with the protein bands in the gel was determined by incubating 0.25-cm gel slices with 10.0 mL of ACS (New England Nuclear) and 0.5 mL of NCS (Amersham) at room temperature in the dark for 2 days. The radioactivity was determined on a Beckman scintillation spectrometer, Model LS-100C.

Acid Gels. Acid–urea gels were prepared by using a modified method of Panyim & Chalkley (1969). Gels were 34 cm long and had a final concentration of 15% acrylamide, 2 M urea, and 0.9 N acetic acid. Gels were preelectrophoresed overnight by using 0.9 N acetic acid at 2 mA per tube (constant current). Samples (75 μg of protein) contained 0.9 N acetic acid, 15% glycerol, 2 M urea, 10% 2-mercaptoethanol, and 0.001% Pyronine Y. Gels were electrophoresed at 190 V (constant voltage) for 26 h at room temperature by using 0.9 N acetic acid as the electrode buffer. Gels were stained

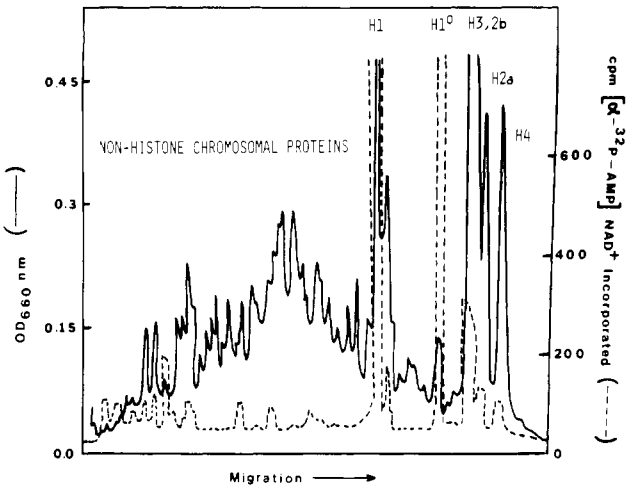

FIGURE 2: $NaDodSO_4$ gel electrophoresis densitometric tracing showing the distribution of radioactivity in the total chromosomal proteins following incubation of isolated rat liver nuclei with [α-^{32}P-AMP]NAD$^+$.

for 2 h in 1% Fast Green FCF in 7% acetic acid. Gels were destained electrically by using 7% acetic acid. Gels were scanned at 630 nm by using a Gilford spectrophotometer (Model 2400) equipped with a gel scanning device. The radioactivity in the gels was determined by cutting the gels into 0.25-cm slices and incubating the slices in 0.3 mL of NCS and 10 mL of scintillation solution Formula 949 (New England Nuclear Corp.) for 2 days. The radioactivity in the samples was determined.

Proof That the Nuclear Proteins Are ADP Ribosylated Following Incubation with [^{3}H]NAD$^+$. To determine whether the various classes of nuclear proteins were ADP ribosylated following incubation of nuclei with [^{3}H]NAD$^+$ or [^{3}H]-2′dNAD$^+$, we extracted the total nuclear proteins, nonhistone proteins, histones, and H1 histones as described earlier. The proteins were incubated with either DNase, RNase, SVPD I, or 0.3 N NaOH according to Nishizuka et al. (1967). After termination of these four reactions, 30% Cl_3AcOH, 4 °C, was added to a final concentration of 20% Cl_3AcOH. The acid-insoluble material was collected on Millipore filters (type HA, 0.45 μm) and washed three times with 20% Cl_3AcOH, 4 °C. The filters were dried under an infrared lamp, and the radioactivity was determined.

Average Chain Length of Poly(ADP-Ribose) Associated with Each of the Classes of Nuclear Proteins. The various classes of nuclear proteins labeled with either [^{3}H]NAD$^+$ or [^{3}H]2′dNAD$^+$ were isolated as described earlier. The chain length of the poly(ADP-ribose) formed was determined (Suhadolnik et al., 1977).

Results

Analysis of HeLa and Rat Liver Histone and Nonhistone Proteins Following ADP Ribosylation with NAD$^+$ and 2′dNAD$^+$. To determine the nature of the nuclear protein acceptor for either ADP-ribose or 2′dADP-ribose, [α-^{32}P-AMP]NAD$^+$ and [α-^{32}P-2′dAMP]2′dNAD$^+$ were incubated with nuclei isolated from rat liver and HeLa cells. The chromosomal proteins were isolated, analyzed by $NaDodSO_4$–polyacrylamide slab gel electrophoresis and acid–urea gels, and examined for covalently bound radioactive 2′dADP-ribose or ADP-ribose.

When isolated rat liver nuclei were incubated with [α-^{32}P-AMP]NAD$^+$, of the total ^{32}P covalently bound to the nuclear proteins, 85% was covalently bound to the histone chromosomal proteins and 15% of the ^{32}P was covalently bound

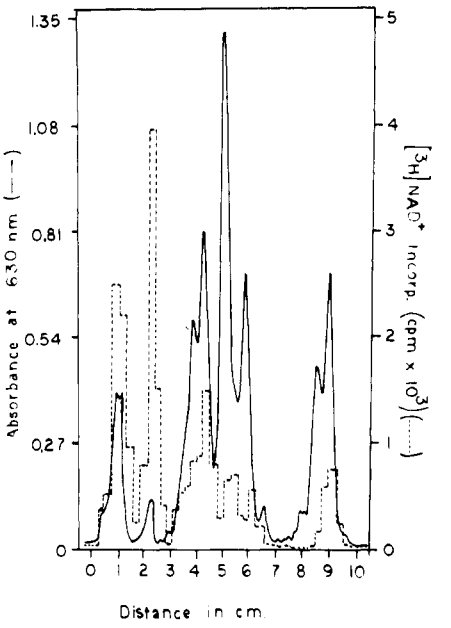

FIGURE 3: Acid–urea gel electrophoresis densitometric tracing showing the distribution of radioactivity in the histone proteins following incubation of isolated rat liver nuclei with [*adenine*-2,8-^{3}H]NAD$^+$.

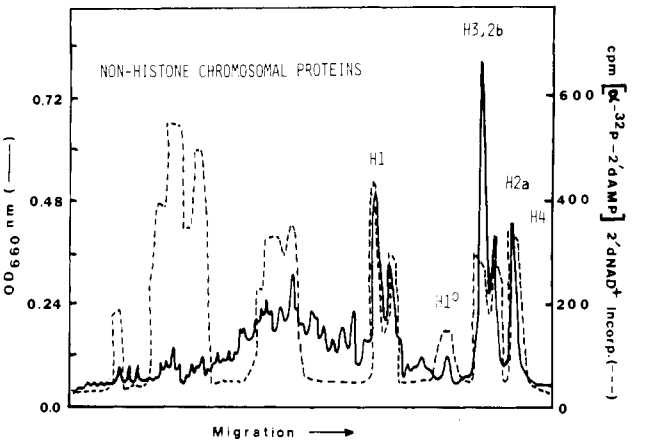

FIGURE 4: $NaDodSO_4$ gel electrophoresis densitometric tracing showing the distribution of radioactivity in the total chromosomal proteins following incubation of isolated rat liver nuclei with [α-^{32}P-2′dAMP]2′dNAD$^+$.

to the nonhistone proteins (Figure 2). Of the radioactivity covalently bound to the histones, 35% was associated with H1; 47% was associated with a protein resembling H1°.

The separation of H1° from other histones has been accomplished by acid–urea polyacrylamide gel electrophoresis (Marks et al., 1975). When isolated rat liver nuclei were incubated with [*adenine*-2,8-^{3}H]NAD$^+$, a protein corresponding to H1° was ADP ribosylated (Figure 3). Of the ^{32}P–histone bound material, 32, 40, 16, 5, 2, and 5% of the ADP-ribose is associated with H1, H1°, H3, H2a, H2b, and H4 histones, respectively.

When isolated rat liver nuclei were incubated with [α-^{32}P-2′dAMP]2′dNAD$^+$, 39% of the incorporated ^{32}P was covalently bound to the histone chromosomal proteins while 61% of the incorporated ^{32}P was covalently bound to the nonhistone proteins. Of the ^{32}P associated with the histones, 35% of the incorporated ^{32}P was associated with H1 and 7% of the ^{32}P was associated with H1° (Figure 4).

When isolated HeLa cell nuclei were incubated with [α-^{32}P-AMP]NAD$^+$, 72% of the incorporated ^{32}P was covalently bound to the histone chromosomal proteins and 28% was

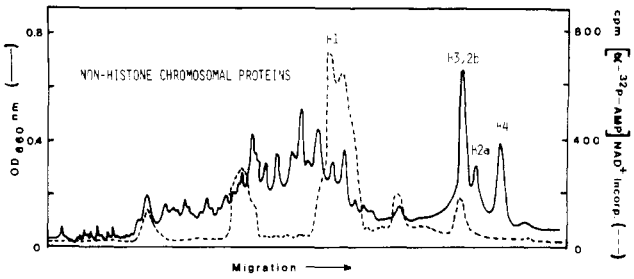

FIGURE 5: $NaDodSO_4$ gel electrophoresis densitometric tracing showing the distribution of radioactivity in the total chromosomal proteins following incubation of isolated HeLa cell nuclei with [α-^{32}P-AMP]NAD^+.

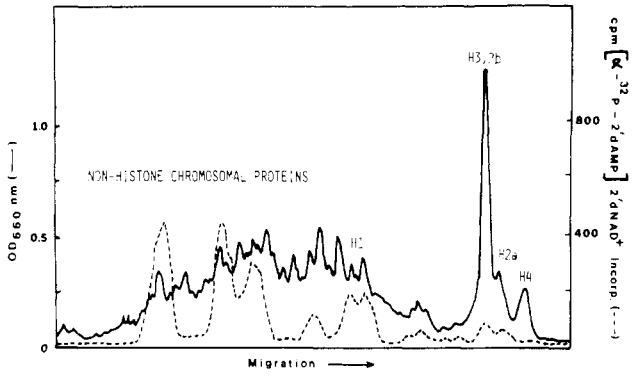

FIGURE 6: $NaDodSO_4$ gel electrophoresis densitometric tracing showing the distribution of radioactivity in the total chromosomal proteins following incubation of isolated HeLa cell nuclei with [α-^{32}P-2′dAMP]2′dNAD$^+$.

covalently bound to the nonhistone proteins (Figure 5). Of the ^{32}P bound to the histone proteins, 73% was associated with H1 and 8% was associated with the H3/H2b peak.

When isolated HeLa cell nuclei were incubated with [α-^{32}P-2′dAMP]2′dNAD$^+$, 25% of the incorporated ^{32}P was covalently bound to the histone chromosomal proteins and 75% of the incorporated ^{32}P was covalently bound to the nonhistone proteins. Of the ^{32}P covalently bound to the histone proteins, 54% was associated with H1 and 32% was associated with the H3/H2b and H2a peaks (Figure 6).

Proof That the Nuclear Proteins Following Incubation with [^{3}H]NAD$^+$ or [^{3}H]2′dNAD$^+$ Are ADP Ribosylated. To prove that the radioactive material associated with histones or nonhistone proteins of nuclei isolated from rat liver following incubation with either [*adenine*-2,8-^{3}H]NAD^+ or [8-^{3}H]-2′dNAD$^+$ was ADP-ribose or 2′dADP-ribose, respectively, we analyzed the radioactive material covalently bound to the nuclear proteins as follows. Poly(ADP-ribose) is resistant to hydrolysis by DNase and RNase, but is hydrolyzed by snake venom phosphodiesterase (Nishizuka et al., 1967). Poly-(ADP-ribose) is readily hydrolyzed from its acceptor protein by 0.3 N NaOH. When analyzed by these four methods, the radioactive material covalently bound to the various classes of rat liver nuclei proteins when [*adenine*-2,8-^{3}H]NAD^+ was incubated with nuclei isolated from rat liver appeared to be poly(ADP-ribose). All of the radioactivity was released by treatment with SVPD I or alkali, but was unaffected by treatment with DNase or RNase (Table I). Similar results were obtained when the acid-precipitable, tritium-labeled material from rat liver nuclei which had been incubated with [8-^{3}H]2′dNAD$^+$ was analyzed (Table II). Similar results were obtained by using HeLa cell nuclei. Results of these studies also indicated that the radioactive material covalently bound to nuclear proteins was ADP-ribose or 2′dADP-ribose (data not shown).

Table I: Evidence That the ADP Ribosylation of the Nuclear Chromosomal Proteins Occurs Following Incubation of Isolated Rat Liver Nuclei with [*adenine*-2,8-^{3}H]NAD^+ [a]

treatment	total nuclear proteins[b] (cpm)	nonhistone proteins[c] (cpm)	histone proteins[d] (cpm)	H1°[e] (cpm)
RNase	2600	650	1960	1530
DNase	2630	666	1900	1560
SVPD I	100	60	80	80
0.3 N NaOH	70	50	60	80

[a] Poly(ADP-ribose) is resistant to hydrolysis by RNase and DNase but is readily hydrolyzed by SVPD I and 0.3 N NaOH. Assays were performed according to Nishizuka et al. (1967). [b] 2600 cpm added per assay. [c] 660 cpm added per assay. [d] 1950 cpm added per assay. [e] 1550 cpm added per assay.

Table II: Evidence That the ADP Ribosylation of the Nuclear Chromosomal Proteins Occurs Following Incubation of Isolated Rat Liver Nuclei with [^{3}H]2′dNAD$^+$ [a]

treatment	total nuclear proteins[b] (cpm)	nonhistone proteins[c] (cpm)	histone proteins[d] (cpm)	H1°[e] (cpm)
DNase	1690	1030	700	270
RNase	1680	1040	720	270
SVPD I	50	40	50	70
0.3 N NaOH	60	70	70	80

[a] Poly(ADP-ribose) is resistant to hydrolysis by RNase and DNase but is readily hydrolyzed by SVPD I and 0.3 N NaOH. Assays were performed according to Nishizuka et al. (1967). [b] 1690 cpm added per assay. [c] 1050 cpm added per assay. [d] 770 cpm added per assay. [e] 270 cpm added per assay.

Table III: Average Chain Lengths of Poly(ADP-Ribose) Associated with Either Histones or Nonhistone Chromosomal Proteins

nuclei from	ADP-ribose from	average chain length associated with: histones	average chain length associated with: nonhistones
rat liver	NAD^+	7.2	1.6
rat liver	2′dNAD$^+$	1.2	1.1
HeLa cells	NAD^+	5.0	1.3
HeLa cells	2′dNAD$^+$	1.2	1.1

Determination of the Average Chain Length of ADP-Ribose from NAD$^+$ and 2′dNAD$^+$ Associated with Each of the Classes of Nuclear Proteins. The average chain lengths of poly(ADP-ribose) from NAD^+ and 2′dNAD$^+$ associated with either the nonhistone or histone in nuclei isolated from rat liver and HeLa cells were determined (Table III). With NAD^+, the nonhistone fractions of nuclei isolated from rat liver and HeLa cells have average chain lengths of 1.2 and 1.5. The average chain lengths of poly(ADP-ribose) released from the histone fractions were 7.2 and 5.0 from rat liver and HeLa nuclei, respectively.

When 2′dNAD$^+$ was incubated with either nuclei isolated from HeLa cells or rat liver, the average chain length associated with either the nonhistone chromosomal proteins or the histone proteins was always equal to or less than 1.2 but always greater than 1.0.

Discussion

The average chain lengths of ADP-ribose vary from 1 to 30 ADP-ribose units. Tanaka et al. (1978) recently reported that poly(ADP-ribose) is not a linear homopolymer as was

originally believed but that the polymer exists as a branched structure. Their observation was based on the discrepancy between the size of the polymer (more than 4.5×10^5 daltons) and average chain length. Their data suggest that there is a linkage other than the long established 1″ → 2′ ribosidic bond. We reported earlier on a similar discrepancy in ADP ribosylation and a 1″ → 3′ ribosidic bond when 2′dNAD$^+$ replaced NAD$^+$ as the substrate for poly(ADP-ribose) polymerase (Suhadolnik et al., 1977). Our data and the data of Tanaka et al. (1978) and Miwa et al. (1979) suggest that the eucaryotic enzyme systems responsible for the synthesis of poly(ADP-ribose) can form linkages other than the 1″ → 2′ ribosidic bond.

Changes in ADP ribosylation of DNA synthesis occur in nuclei isolated from normal and neoplastic tissues following the addition of NAD$^+$ structurally modified in the ribosyl moiety of the adenosine of NAD$^+$ (Suhadolnik et al., 1977). ADP ribosylation from 2′dNAD$^+$ and 3′dNAD$^+$ results in the formation of monomers and dimers instead of polymers with an average chain length of 10 when NAD$^+$ is the substrate. Furthermore, 2′dNAD$^+$ and 3′dNAD$^+$, at 0.5 mM, are about 10 times more effective as inhibitors of DNA synthesis in nuclei isolated from rat liver than is NAD$^+$. With nuclei isolated from HeLa cells, NAD$^+$ does not inhibit DNA synthesis, whereas the analogue, 2′dNAD$^+$, does inhibit DNA synthesis. These marked differences in poly(ADP ribosylation) and inhibition of DNA synthesis prompted the design of experiments to explain why 2′dNAD$^+$ is a better inhibitor of DNA synthesis in nuclei isolated from normal and neoplastic cells.

This study describes the effects of NAD$^+$ and 2′dNAD$^+$ on the posttranscriptional modification by ADP ribosylation of histone and nonhistone proteins of nuclei from rat liver and HeLa cells. When nuclei isolated from rat liver were incubated with [α-^{32}P]NAD$^+$, the majority of [^{32}P]ADP-ribose was associated with the histone fraction. Seventy percent of the incorporated ^{32}P associated with rat liver histones was covalently linked to the H1 species. This is in agreement with Riquelme et al. (1977). Of the incorporated ^{32}P covalently bound to histones, 40% was associated with a protein which appeared to be H1°, a minor lysine-rich histone which is present only in tissues with very low rates of cell division (Marks et al., 1975; Varricchio, 1977). The migration of the reputed H1° in the acid–urea gel system, as well as its extraction by perchloric acid, strongly suggests that the protein is H1°. Recent reports that HMG17 protein would electrophorese similarly to H1° in an NaDodSO$_4$ gel (Mathew et al., 1979) or in an acid–urea gel (Spiker et al., 1978) may mean that the putative H1° may be an HMG species. Further experimentation is necessary to establish the nature of this protein. Caplan has described experiments that show the ADP ribosylation of HMG's (Caplan et al., 1978).

Incubations of isolated rat liver nuclei with [α-^{32}P-2′dAMP]2′dNAD$^+$ show a marked difference in the ^{32}P labeling pattern of nuclear proteins when compared to labeled NAD$^+$ (Figures 2 and 4; Figures 5 and 6). There is a preferential 2′dADP ribosylation of the nonhistone proteins by 2′dNAD$^+$. Because the nonhistone proteins include many of the enzymes necessary for DNA synthesis and chromatin structure, the increase in 2′dADP ribosylation of the nonhistone proteins may explain why 2′dNAD$^+$ is a much more potent inhibitor of DNA synthesis than is NAD$^+$.

When nuclei isolated from HeLa cells were incubated with [α-^{32}P-AMP]NAD$^+$, most of the ^{32}P was associated with the histones and 73% of the ^{32}P covalently bound to the histones was associated with the H1 histones. This value is similar to the report of Tanuma et al. (1977). Although little or no H1° has been isolated from HeLa cells, 7% of the ^{32}P resides in the H1° region of the gel (Marks et al., 1975). Because H1° is present primarily in nonproliferating, resting tissues, and because HeLa cells are rapidly proliferating, H1° would not be an expected protein.

Examination of the radioactivity associated with the nonhistone and histone proteins when nuclei isolated from HeLa cells were incubated with [α-^{32}P-2′dAMP]2′dNAD$^+$ revealed a similar labeling pattern to that observed when nuclei isolated from rat liver were incubated with 2′dNAD$^+$. Most of the ^{32}P was covalently bound to the nonhistone proteins.

One implication of the radioactive labeling patterns of the histone and nonhistone proteins is as follows. The observation that histones, specifically H1 histones, are the main target for ADP ribosylation when NAD$^+$ is incubated with isolated nuclei suggests that ADP ribosylation, like phosphorylation of nuclear proteins, may function in structural perturbations of chromatin.

An analogy may exist between the phosphorylation of H1 and the ADP ribosylation of H1. The phosphorylation of H1 begins in G_1 phase to prepare the cell for DNA replication. At the same time the phosphorylation of H1 commences, there is an increase in the amount of DNA associated with the membrane lipoprotein (Bradbury et al., 1973). H1 phosphorylation may organize chromatin and condense it into chromosomes. This change in chromatin structure allows an increase in the association of the chromatin with the membrane lipoprotein. The phosphorylation and dephosphorylation of H1 may allow changes in the chromatin structure that permit cells to enter the replicative phase of the cell cycle, DNA to be replicated during S phase, and chromosomes to be condensed and segregated during mitosis.

In HeLa cells, poly(ADP-ribose) polymerase activity is localized within the internucleosomal "linker" regions of HeLa cell chromatin (Giri et al., 1978). ADP ribosylation occurs primarily at the H1-rich nucleosome region, and little or no ADP ribosylation is detected on nucleosome bodies (Smulson et al., 1976). Poly(ADP-ribose) has been reported to cross-link HeLa histone proteins. Since the amount of poly(ADP-ribose) reaches a maximum at the S/G_2 boundary, the cross-linking of two histones by poly(ADP-ribose) might function to link widely spaced H1 molecules along internucleosome regions and condense chromatin. The reversibility of the chromatin condensation could occur by degradation of the polymer by poly(ADP-ribose) glycohydrolase (Lorimer et al., 1977; Stone et al., 1978).

Histones derived from nuclei preincubated with NAD$^+$ have less affinity for DNA than non-ADP-ribosylated histones. Covalent attachment of poly(ADP-ribose) to histones may alter histone structure by introducing a cluster of negative charges into the highly basic carboxy-terminal region of the molecule and may weaken the interactions between histone H1 and DNA in the transcriptionally active region (Smulson et al., 1976; Yoshihara & Koide, 1973).

Smulson (1975) reported that the ADP ribosylation of histone in HeLa cells is high in G_1 and low in S phase. ADP ribosylation of HeLa nuclear proteins leads to an enhancement rather than to an inhibition in the number of primer sites for DNA polymerase. Sudhaker et al. (1979) have expanded our knowledge of chromatin. They showed that nitrosoureas differ in their effects on poly(ADP ribosylation) and interactions with chromatin-associated proteins.

It is conceivable that ADP ribosylation of H1 by NAD$^+$ explains the enhanced DNA synthesis in HeLa cells. This does

not explain the decrease in DNA synthesis observed in normal rat liver. The decrease in DNA synthesis may be due to ADP ribosylation of a DNA endonuclease in rat liver nuclei. This DNA endonuclease may be inactive or absent in HeLa cells. Another possibility is that H1°, which is present in resting tissues but not in rapidly dividing tissues, exerts negative control on DNA synthesis in rat liver. ADP ribosylation may render H1° inactive as a repressor of enzymes necessary for DNA synthesis. In nuclei isolated from HeLa cells and rat liver, incubation of 2′dNAD$^+$ caused an inhibition of DNA synthesis and a considerable increase in the 2′dADP ribosylation of nonhistone proteins as compared to the ADP ribosylation with NAD$^+$. Nonhistone chromosomal proteins contain many of the enzymes necessary for DNA and RNA synthesis and those enzymes which phosphorylate histones and nonhistones and acetylate and methylate histones. The nonhistone fraction also contains the poly(ADP-ribose) glycohydrolase (Miyakawa et al., 1972). Several distinct HeLa and rat liver nonhistone proteins were 2′dADP ribosylated by 2′dNAD$^+$ (Figures 4 and 6). The ADP ribosylation of one or more of these proteins by 2′dNAD$^+$ may well explain the inhibition of DNA synthesis in nuclei isolated from HeLa cells and rat liver. Current studies, to elucidate the identity of these nonhistone protein acceptors of short chains of 2′dADP-ribose, are under investigation.

Upon incubation of NAD$^+$ with nuclei isolated from either rat liver or HeLa cells, the average chain length of the poly(ADP-ribose) formed was always less than 2 for the nonhistone proteins. Average chain lengths of poly(ADP-ribose) for histones were 7.2 and 5.0 for rat liver and HeLa histones, respectively. Mono-ADP ribosylation is high in resting tissues such as rat liver and low in rapidly dividing tissues such as neonatal rat liver or Zajdel hepatoma (Stone et al., 1976). Stone & Hilz (1975) measured the endogenous levels of mono(ADP-ribose) residues in rat liver and found nearly equal amounts. This indicates a higher level of acceptor sites for mono(ADP-ribose) residues than for polymer chains. Only a limited amount of 2′dNAD$^+$ can form dimers of 2′dADP-ribose; 88% of the 2′dADP-ribose covalently bound to nuclear proteins exists as the monomer (Suhadolnik et al., 1977). Because NAD$^+$ forms long polymers whereas 2′dNAD$^+$ forms monomers or dimers, 2′dADP-ribose can bind covalently with more protein acceptor sites than can ADP-ribose. This phenomenon may also account for the 10-fold increase in inhibition of DNA synthesis observed when nuclei isolated from rat liver are incubated with 2′dNAD$^+$ instead of NAD$^+$ (Hershey et al., 1973). It may also explain why 2′dNAD$^+$ inhibits DNA synthesis in nuclei isolated from Novikoff tumors and fetal rat livers, whereas NAD$^+$ is ineffective as an inhibitor of DNA synthesis in these same tissues (Suhadolnik et al., 1977).

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References

Adamietz, P., Phillips, C., & Hilz, H. (1976) *Proceedings of the 4th International Symposium on Poly(ADP-Ribose) and ADP-Ribosylation of Proteins*, p 3, Walter de Gruyter, Berlin.

Blobel, G., & Potter, V. R. (1966) *Science 154*, 1662–1665.

Bonner, W. M. (1975) *J. Cell Biol. 64*, 421–430.

Bradbury, E. M., Carpenter, B. G., & Rattle, H. W. E. (1973) *Nature (London) 241*, 123–126.

Burzio, L., & Koide, S. S. (1970) *Biochem. Biophys. Res. Commun. 40*, 1013–1020.

Burzio, L., & Koide, S. S. (1973) *Biochem. Biophys. Res. Commun. 53*, 572–579.

Burzio, L. O., Riquelme, P. T., & Koide, S. S. (1979) *J. Biol. Chem. 254*, 3029–3037.

Caplan, A. I., & Rosenberg, M. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1852–1857.

Chambon, P., Weill, J. D., & Mandel, P. (1963) *Biochem. Biophys. Res. Commun. 11*, 29–43.

Colyer, R. A., Burdette, K. E., & Kidwell, W. R. (1973) *Biochem. Biophys. Res. Commun. 53*, 960–966.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Ferris, G. M., & Clark, J. B. (1971) *Biochem. J. 121*, 655–662.

Fujimura, S., Hasegawa, S., Shimizu, Y., & Sugimura, T. (1967) *Biochim. Biophys. Acta 145*, 247–259.

Giri, C. P., West, M. H. P., & Smulson, M. (1978) *Biochemistry 17*, 3495–3500, 3501–3505.

Hayaishi, O., & Ueda, K. (1977) *Annu. Rev. Biochem. 46*, 95–116.

Hershey, H. V., Stieher, J. F., & Mueller, G. C. (1973) *Eur. J. Biochem. 34*, 383–394.

Keller, E. G. (1964) *Biochem. Biophys. Res. Commun. 17*, 412–415.

Kidwell, W. R., & Burdette, K. E. (1974) *Biochem. Biophys. Res. Commun. 61*, 766–773.

Laemmli, U., & Favre, M. (1973) *J. Mol. Biol. 80*, 575–589.

Lorimer, W. S., III, Stone, P. R., & Kidwell, W. R. (1977) *Exp. Cell Res. 106*, 261–266.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marks, D. B., Kanefsky, T., Keller, B. J., & Marks, A. D. (1975) *Cancer Res. 35*, 886–889.

Mathew, C. G. P., Goodwin, G. H., & Johns, E. W. (1979) *Nucleic Acids Res. 6*, 167–179.

Miwa, M., Saikawa, N., Yamaizumi, Z., Nishimura, S., & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 595–599.

Miyakawa, N., Ueda, K., & Hayaishi, O. (1972) *Biochem. Biophys. Res. Commun. 49*, 239–245.

Morton, R. K. (1958) *Nature (London) 181*, 540–542.

Müller, W. E. G., & Zahn, R. K. (1976) *Mol. Cell Biochem. 12*, 147–158.

Müller, W. E. G., Totsuka, A., Nusser, I., Obermeier, J., Rohde, H. J., & Zahn, R. K. (1974) *Nucleic Acids Res. 1*, 1317–1327.

Nishizuka, Y., Ueda, K., Nakazawa, K., & Hayaishi, O. (1967) *J. Biol. Chem. 242*, 3164–3171.

Nishizuka, Y., Ueda, K., Honjo, T., & Hayaishi, O. (1968) *J. Biol. Chem. 243*, 3765–3767.

Okayama, H., & Hayaishi, O. (1978) *Biochem. Biophys. Res. Commun. 84*, 755–762.

Okazaki, H., Niedergang, C., & Mandel, P. (1976) *FEBS Lett. 62*, 255–258.

Ord, M. G., & Stocken, L. A. (1977) *Biochem. J. 161*, 583–592.

Otake, H., Miwa, M., Fujimura, S., & Sugimura, T. (1969) *J. Biochem. (Tokyo) 65*, 145–146.

Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.

Proctor, N. H., & Casida, J. E. (1975) *Science 190*, 580–582.

Riquelme, P. T., Burzio, L. O., & Koide, S. S. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 785.

Roberts, J. H., Stark, P., & Smulson, M. (1973) *Biochem. Biophys. Res. Commun. 52*, 43–50.
Roberts, J. H., Stark, P., Giri, C. P., & Smulson, M. (1975) *Arch. Biochem. Biophys. 171*, 305–315.
Schwartz, J. P., Passoneare, J. V., Johnson, G. S., & Pastan, I. (1974) *J. Biol. Chem. 249*, 4138–4143.
Shima, T., Hasegawa, S., Fujimura, S., Matsubara, H., & Sugimura, T. (1969) *J. Biol. Chem. 244*, 6632–6635.
Smulson, M. (1975) *Science 188*, 1223–1224.
Smulson, M. E., & Rideau, C. (1972) *Biochim. Biophys. Acta 111*, 408–416.
Smulson, M., & Roberts, J. H. (1973) *Poly(ADP-Ribose), Int. Symp., 1973*, 141.
Smulson, M., Giri, C., Sudhakar, S., & Mullins, D. (1976) in *Proceedings of the 4th International Symposium on Poly(ADP-Ribose) and ADP-Ribosylation of Proteins* (Hilz, H., Ed.) p 7, Walter de Gruyter, Berlin.
Spiker, S., Mardian, J. K. W., & Isenberg, I. (1978) *Biochem. Biophys. Res. Commun. 82*, 129–135.
Stone, P. R., & Hilz, H. (1975) *FEBS Lett. 57*, 209–212.
Stone, P. R., Bredehorst, R., Kittler, M., Lengyel, H., & Hilz, H. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 51–56.
Stone, P. R., Lorimer, W. S., III, Ranchalis, J., Danley, M., & Kidwell, W. R. (1978) *Nucleic Acids Res. 5*, 173–184.
Sudhakar, S., Tew, K. D., Schein, P. S., Woolley, P. V., & Smulson, M. E. (1979) *Cancer Res. 39*, 1411–1417.
Suhadolnik, R. J., Baur, R., Lichtenwalner, D. M., Uematsu, T., Roberts, J. H., Sudhakar, S., & Smulson, M. (1977) *J. Biol. Chem. 252*, 4134–4144.
Tanaka, M., Hayashi, K., Sakura, H., Miwa, M., Matsushima, T., & Sugimura, T. (1978) *Nucleic Acids Res. 5*, 3183–3194.
Tanuma, S., Emoto, T., & Yamada, Y. (1977) *Biochem. Biophys. Res. Commun. 74*, 599–605.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.
Ueda, K., Omachi, A., Kawaichi, M., & Hayaishi, O. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 205–209.
Varricchio, F. (1977) *Arch. Biochem. Biophys. 179*, 715–717.
Wintzerith, M., Klein, M., Mandel, L., & Mandel, P. (1961) *Nature (London) 191*, 467–469.
Yoshihara, K., & Koide, S. S. (1973) *FEBS Lett. 35*, 261–264.
Yoshihara, K., Tanigawa, Y., Burzio, L., & Koide, S. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 289–293.

Purification and Characterization of the Crown Gall Specific Enzyme Nopaline Synthase†

John D. Kemp,* Dennis W. Sutton, and Ethan Hack

ABSTRACT: Nopaline synthase of sunflower (*Helianthus annuus* L.) crown gall tissue induced by *Agrobacterium tumefaciens* strain C58 or T37 (nopaline utilizers) was purified to homogeneity as judged by analytical disc gel electrophoresis. The native enzyme elutes from a column of Ultrogel AcA 34 as a single peak with an estimated molecular weight of 158 000. The dissociated enzyme migrates on $NaDodSO_4$–polyacrylamide gels as a single band with a molecular weight of 40 000. Thus, the native enzyme appears to be composed of four equal-weight subunits. Nopaline synthesizing activity is found exclusively in crown gall tissues induced by strains of *A. tumefaciens* that utilize nopaline (e.g., C58 and T37). We found the same tissue specificity for the purified protein that we believe represents nopaline synthase. The results of kinetic studies of the purified enzyme are consistent with a ter-bi rapid-equilibrium random-order mechanism. Nopaline synthase is probably responsible for the in vivo synthesis of both N^2-(1,3-dicarboxypropyl)arginine (nopaline) and N^2-(1,3-dicarboxypropyl)ornithine (ornaline) in crown gall tissues since substrate specificities and K_m values do not change during purification.

Cells of a variety of dicotyledonous plants are transformed to crown gall tumor cells by transfer of DNA from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* (E. F. Sm. and Town.) Conn. to the host (Chilton et al., 1977). Although Ti plasmids are quite large [(112–156) × 10^6 daltons] (Zaenen et al., 1974), only a relatively small portion of their DNA [(3–5) × 10^6 daltons] is stably associated with tumor cells (Chilton et al., 1977). The transferred DNA (T-DNA) appears to be functional, since several investigators (Drummond et al., 1977; Gurley et al., 1979; Ledeboer, 1978) have demonstrated that tumor tissue synthesizes RNA that hybridizes to T-DNA. However, the question of whether the RNA transcribed from T-DNA actually codes for structural gene products is still unresolved.

Tumors incited by most strains of *A. tumefaciens* can be assigned to two groups (octopine type and nopaline type) based on the crown gall specific amino acid derivatives that they synthesize. Octopine-type tumors synthesize N^2-(1-carboxyethyl) amino acid derivatives of arginine (octopine) (Mênagê & Morel, 1964), of lysine (lysopine) (Biemann et al., 1960), of ornithine (octopinic acid) (Goldmann-Mênagê, 1970), and of histidine (histopine) (Kemp, 1977). The nopaline-type tumors, in contrast, synthesize N^2-(1,3-dicarboxypropyl)arginine (nopaline) (Goldmann-Mênagê, 1970) and N^2-(1,3-dicarboxypropyl)ornithine (ornaline) (Kemp, 1978). It is the Ti plasmid, not the plant species, that determines whether N^2-(1-carboxyethyl) amino acids or N^2-(1,3-dicarboxypropyl) amino acids are synthesized by the plant tumor (Petit et al., 1970; Bomhoff et al., 1976; Montoya et al., 1977).

The obvious candidates for structural gene products of T-DNA are the enzymes that synthesize the unusual amino

† From the Plant Disease Resistance Research Unit, Agricultural Research, U.S. Department of Agriculture, and the Department of Plant Pathology, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706. *Received March 9, 1979.*

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