Vol. 84, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1978 Pages 537-543

POLY(ADENOSINE DIPHOSPHATE RIBOSE) SYNTHESIS BY ISOLATED NUCLEI OF XENOPUS LAEVIS EMBRYOS: IN VITRO ELONGATION OF IN VIVO SYNTHESIZED CHAINS*

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Received July 21,1978

ABSTRACT

We have studied the synthesis of poly(ADP-ribose) by nuclei isolated from Xenopus laevis embryos at different stages of development. Determination of the total chain length of poly(ADP-ribose) molecules by hydroxylapatite column chromatography generally gave higher values than when the radioactive portions of these molecules, synthesized in vitro, were measured by poly(ethyleneimine)-cellulose thin layer chromatography, after snake venom phosphodiesterase digestion. The results show that most of the poly(ADP-ribose) synthesized in vitro is a covalent elongation of molecules previously initiated in vivo.

INTRODUCTION

A preliminary communication (1) describes our observations on the activity of poly(ADP-ribose)polymerase in vitro during the development of Xenopus laevis embryos, and a more detailed report is in preparation. In this paper we describe the results of measuring the chain lengths of poly(ADP-ribose) molecules attached to protein by two commonly employed procedures. Hydroxylapatite chromatography was used to measure total chain length (2) and PEI-cellulose thin layer chromatography (3) of the products of the degradation by snake venom phosphodiesterase was used to determine the length of the chain synthesized only in vitro. Digestion of the poly(ADP-ribose) with this enzyme produces 5'-AMP from the end of the chains not attached to the acceptor proteins, and 2'-(5"-phosphoribosyl)-5'-AMP(PR-AMP), from the internal residues. The ratio of total radioactivity to that in 5'-AMP gives a measure of the average chain length of the population of molecules (4).

Most previous reports on the chain length of poly(ADP-ribose) present in nuclei incubated with radioactive NAD^+ have generally utilized only the snake

* A preliminary account of these studies was presented at the 36th Meeting of the British Society for Developmental Biology (1977).

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0006-291X/78/0843-0537$01.00/0

venom phosphodiesterase method described above, which consequently gives a measurement only of the average chain length of the components synthesized *in vitro* (5, 6, 7, 8, 9). In recent work using isolated Hela-cell nuclei incubated with [^{3}H]NAD$^+$ Stone *et al* (10) measured the chain length of poly(ADP-ribose) attached to histone H1 by three different procedures, including hydroxylapatite column chromatography and snake venom phosphodiesterase treatment. They obtained similar chain length values from each of the three methods which showed that poly(ADP-ribose) attached to histone H1 was synthesized entirely *in vitro*.

Our work with *Xenopus laevis* nuclei shows that most of the poly(ADP-ribose) synthesized *in vitro* is a covalent extension of chains previously initiated *in vivo*. The possible implications of this novel observation are discussed below.

MATERIALS AND METHODS

Materials: [^{14}C]NAD$^+$: Nicotinamide [U-^{14}C]adenine dinucleotide, ammonium salt (300mCi/mol, 25μCi/ml), was purchased from the Radiochemical Centre, Amersham, Buckinghamshire; Hydroxylapatite was from Bio-Rad Laboratories, Richmond, California, U.S.A.; PEI-cellulose sheets from Brinkman Instruments, New York, U.S.A. and Sephadex G-25 (fine) from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Adult *Xenopus laevis* and chorionic gonadotropin were from Harris's Biological Supplies Ltd., U.K.

Determination of poly(ADP-ribose) chain length by hydroxylapatite column chromatography: Isotopically labelled poly(ADP-ribose) was prepared by incubating partially purified poly(ADP-ribose)polymerase from stage 28 embryos (The enzyme extracted from embryos with 200mM sodium phosphate, pH 8.0, followed by precipitation from this with 20% (w/v) ammonium sulphate), with 15μg of highly polymerized calf thymus DNA and 15μg of calf thymus histones for 30 min at 25°C. The incubation mixture contained 10mM Tris-HCl pH 8.0 at 25°C, 20mM $MgCl_2$, 3.0mM NaF, 1.0mM dithiothreitol and 16.6μM [^{14}C]NAD$^+$ (250mCi/mmol), in a total volume of 200μl. The reaction was terminated by the addition of an equal volume of 40% (w/v) trichloroacetic acid containing 1% (w/v) nicotinamide and 2% (w/v) neutralized tetra-sodium pyrophosphate. After standing on ice about 45 min samples were centrifuged at 8000g for 5 min. The pellets were washed twice with 20% (w/v) trichloroacetic acid containing 1% (w/v) nicotinamide and 2% (w/v) neutralized tetra-sodium pyrophosphate, and twice with 95% (v/v) ethanol. The glycosidic bonds between poly(ADP-ribose) and the acceptor proteins (calf thymus histones) were hydrolyzed by incubating the samples with 50μl of 0.1M NaOH for 60 min at 37°C. 10μl of 0.5M HCl was then added to neutralize the NaOH. 60μl of 50mM Tris-HCl, pH 6.8 at 37°C, containing 20mM $MgCl_2$ and 50μg of pancreatic deoxyribonuclease I were added to the samples which were then incubated for 60 min at 37°C to degrade the DNA. This was followed by a further 60 min incubation, after adding 30μl of water containing 50μg of proteinase K, in order to degrade the proteins. Each sample (150μl), was applied to a column of hydroxylapatite (1cm x 0.6cm) previously equilibrated with 1mM potassium phosphate buffer pH 6.8. Samples were eluted with a 20ml step-gradient of phosphate buffer (1 to 500mM) in 0.5ml fractions. The molarity of the eluted buffer in each fraction was determined by refractive index measurements. 100μl portions of each fraction were then diluted to 1.0ml with distilled water and counted for radioactivity in 10ml of toluene-Triton X-100 scintillation fluid. The efficiency of counting was 90%. The chain length of the poly(ADP-ribose) eluted in each fraction was then determined by degrading it with snake venom phospho-

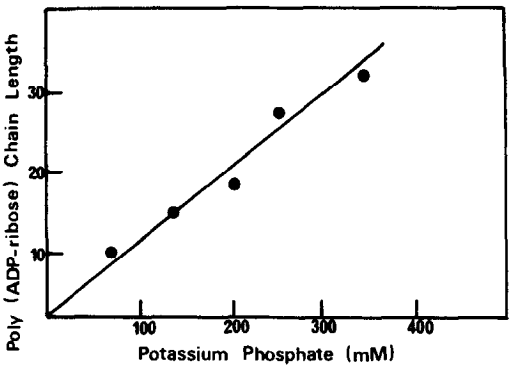

Fig. 1. Poly(ADP-ribose) chain length calibration curve. The numbers on the ordinate refer to the number of ADP-ribose residues.

diesterase followed by PEI-cellulose chromatography of the products (see below). The calibration curve of the relationship between chain length and molarity of the eluting buffer is shown in Fig. 1.

The chain lengths of poly(ADP-ribose) molecules synthesized by the isolated nuclei of Xenopus laevis embryos were measured as described above except that 5×10^6 nuclei were incubated with [^{14}C] NAD^+ instead of the partially purified polymerase, calf thymus DNA and histones. These nuclei were isolated by the method of Farzaneh and Pearson (11).

When fractions eluted from hydroxylapatite were rechromatographed more than 85% of the radioactivity was routinely eluted by the same concentrations of the phosphate buffer.

Desalting of poly(ADP-ribose) samples for chain length determination by PEI-cellulose chromatography: Fractions from the hydroxylapatite columns which contained poly(ADP-ribose) were desalted by a modification of the method of Dancis (12). 7ml of a slurry of Fine Sephadex G-25, previously swelled in distilled water, was poured into a 5ml plastic syringe containing a teflon disc at the bottom. The syringe was placed inside a 10ml conical glass centrifuge tube so that it was suspended from the rim of the tube. This complex was centrifuged at 60g for 5 min, and then for 2 min at 1000g. The column was then washed twice with 4.0ml portions of distilled water and centrifuged at 1000g for 2 min. 400-500µl of fractions collected from hydroxylapatite columns were now applied to the Sephadex columns which were centrifuged at 60g for 5 min and then at 1000g for 2 min, they were then eluted with a further 200µl of distilled water as described above. The desalted poly(ADP-ribose) was collected in the glass centrifuge tube. The recovery was about 95% for long polymers (eluted from hydroxylapatite with 200-500mM phosphate buffer) and 83% for shorter polymers (eluted from hydroxylapatite with 50-200mM phosphate buffer). Material eluted from hydroxylapatite with 1-50mM phosphate buffer was not desalted. The desalting was about 99% efficient as determined by refractive index measurements.

PEI-cellulose thin layer chromatography: The desalted poly(ADP-ribose) was freeze-dried and subsequently digested for 60 min at 37°C with 50µg of snake venom phosphodiesterase in 50µl of 50mM Tris-HCl, pH 6.8 at 37°C, containing 20mM $MgCl_2$. The products were then chromatographed on PEI-cellulose strips as described by Stone et al (3). Only the specific degradation products, 5'-AMP and PR-AMP were detected, showing that the phosphordiesterase used was free from contaminating enzymes. The average chain length of poly(ADP-ribose) in each sample was calculated from the ratio of total radioactivity to that in 5'-AMP (4).

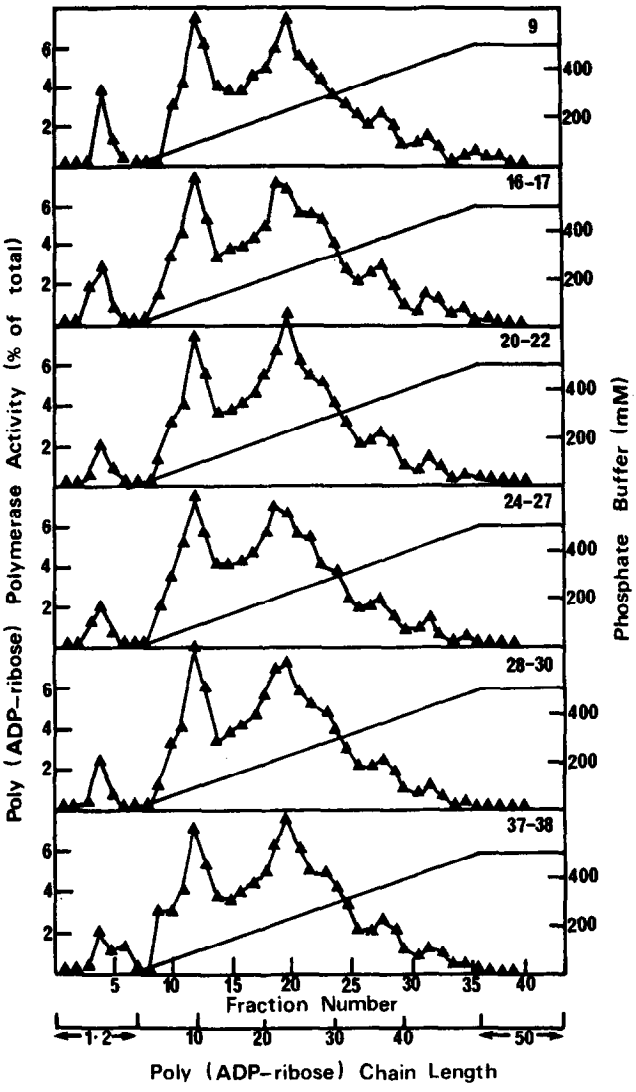

Fig. 2. Poly(ADP-ribose) chain length determination by hydroxylapatite column chromatography. The figure shows the elution profiles of poly(ADP-ribose) molecules obtained from nuclei isolated from embryos at different stages of development (13)(Indicated by the numbers in the top right corner of each figure).

The reproducibility of this procedure for measuring the average chain length of poly(ADP-ribose) molecules was as follows (figures show the average number of ADP-ribose units ± S.D.; figures in parenthesis give the number of determinations carried out): 2.41 ± 0.39 (6), 6.04 ± 0.76 (5), 12.43 ± 1.83 (6), and 19.37 ± 2.42 (4).

RESULTS AND DISCUSSION

Determination of the chain length of poly(ADP-ribose) by hydroxylapatite column chromatography: Fig. 2. shows the elution profiles of poly(ADP-ribose)

molecules synthesized during a 15 min incubation *in vitro* by nuclei isolated from embryos at different stages of development. Since only radioactive molecules could be detected, the chain length measurements by this procedure give the total chain length of the radioactive molecules i.e. those which were synthesized either *in vitro*, or were an elongation *in vitro* of molecules previously initiated *in vivo*.

Determination of the chain length of poly(ADP-ribose) by PEI-cellulose chromatography: The average chain lengths of poly(ADP-ribose) molecules which were eluted from hydroxylapatite with 1mM, 60-100mM, 220-260mM, 380-420mM, and 460-500mM potassium phosphate buffer were determined by PEI-cellulose chromatography after degrading them with snake venom phosphodiesterase. This procedure detects only the radioactive moieties of the molecules, therefore, for a molecule synthesized entirely *in vitro* the chain length value obtained from this and the hydroxylapatite method would be similar, whereas for a molecule previously synthesized partially *in vivo* and subsequently extended covalently *in vitro*, the two techniques would give different chain length values.

In all cases we found that the chain lengths measured by the PEI-cellulose method were either shorter than or equal to those measured by hydroxylapatite chromatography. We interpret this to mean that much of the poly(ADP-ribose) synthesized *in vitro* was a covalent extension of molecules previously initiated *in vivo*. The difference between the chain length of a population of molecules determined by the two methods was used to estimate the proportion of such molecules which were synthesized *in vivo* (Fig. 3).

It is apparent that there are differences in the proportions of the parts of the molecules synthesized *in vitro* and *in vivo*. For example, between embryonic stages 20 and 27 the 10-unit long poly(ADP-ribose) molecules are synthesized almost entirely *in vitro*, whilst more than 8 units from such a molecule is synthesized *in vivo* in stage 37-38 embryos. Similarly, whilst at stage 20-22 only 15 units of a 42-unit long molecule are synthesized *in vivo*, at the later stage of 24-27, 38 units are synthesized *in vivo*.

Although it is not possible to assign functions to these changes in chain length at this time, the results have some interesting implications. They show for the first time, that elongation *in vitro* of poly(ADP-ribose) molecules previously synthesized *in vivo* can occur. Survival of these *in vivo* chains through the experimental procedures implies that nuclei isolated from *Xenopus* embryos contain little activity of enzymes which degrade poly(ADP-ribose), such as poly(ADP-ribose)glycohydrolase, or that these enzymes are inactive throughout the experimental conditions (11).

The presence of some poly(ADP-ribose) molecules synthesized entirely *in vitro* is presumably due to *de novo* initiation, but whether the acceptor proteins in

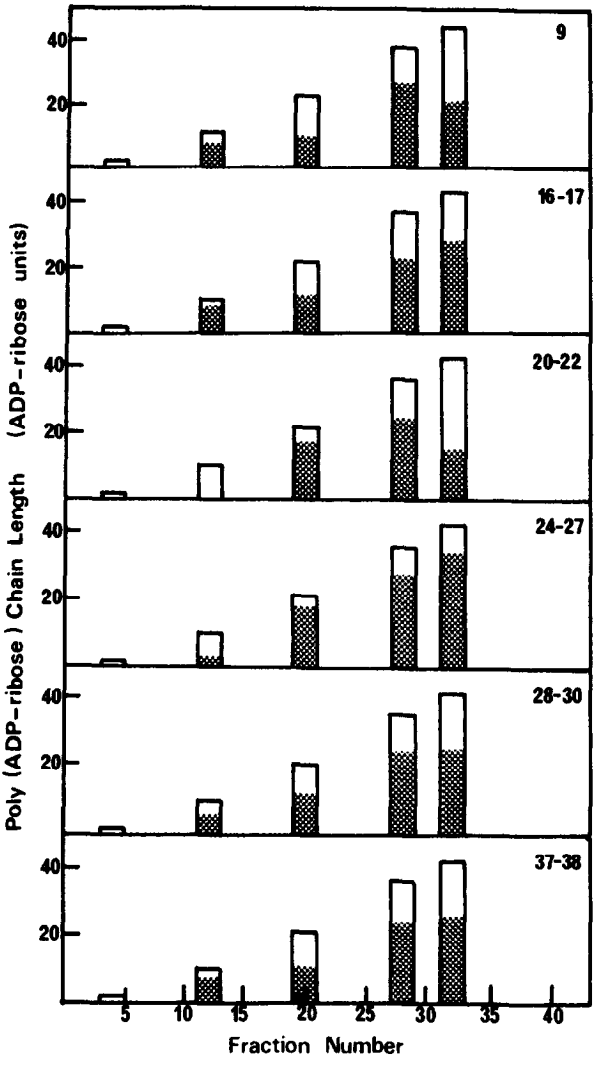

Fig. 3. Poly(ADP-ribose) chain length: proportions synthesized *in vitro* and *in vivo*. The histograms show the total chain lengths determined by hydroxylapatite column chromatography, the radioactive portions synthesized *in vitro* (□) and the presumed portions synthesized *in vivo* (■), calculated from the total chain length minus the portion synthesized *in vitro*.

these cases are the natural acceptors or are simply artifacts of the *in vitro* conditions is unclear. This latter possibility, if correct, casts doubt on the validity of many *in vitro* studies if the conclusions from such work are to be extrapolated to the *in vivo* situation. In this context we think that this *Xenopus in vitro* system offers a means of investigating the nature of proteins which are naturally ADP-ribosylated, that is, those proteins containing residues

covalently extended *in vitro*, with a view to ascertaining potential biological roles for this interesting process of protein modification.

ACKNOWLEDGEMENTS: We are grateful to the Medical Research Council for their support. We acknowledge the advice and assistance of our colleagues Miss Grainne MacDermott and Mr. Henry M. Furneaux of this laboratory, and of Professor Sydney Shall (University of Sussex) for helpful comments during the preparation of the manuscript. We would also like to thank Dr. J. McKenzie (Department of Developmental Biology, University of Aberdeen) for his interest and encouragement throughout this work.

REFERENCES

1. Farzaneh, F. and Pearson, C.K. (1977) Biochem. Soc. Trans. 5, 733-734.
2. Sugimura, T.; Yoshimura, N.; Miwa, M.; Nagai, H. and Nagao, M. (1971) Arch. Biochem. Biophys. 147, 660-665.
3. Stone, P.R.; Whish, W.J.D. and Shall, S. (1973) FEBS Lett. 36, 334-337.
4 Nishizuka, Y.; Ueda, K.; Yoshihara, K.; Yamamura, H.; Takeda, M.; Hayaishi, O. (1969) Cold Spr. Harb. Symp. Quant. Biol. 34, 781-786.
5. Lehmann, A.R.; Kirk-Bell, S.; Shall, S. and Whish, W.J.D. (1974) Exp. Cell Res. 83, 63-72.
6. Brightwell, M.D.; Leech, C.E.; O'Farrell, M.K.; Whish, W.J.D. and Shall, S. (1975) Biochem. J. 147, 119-129.
7. Burzio, L.; Reich, L. and Koide, S.S. (1975) Proc. Soc. Exp. Biol. (N.Y.) 149, 933-938.
8. Rickwood, D.; MacGillivray, A.J. and Whish, W.J.D. (1977) Eur. J. Biochem. 79, 589-598.
9. Lorimer III, W.S.; Stone, P.R. and Kidwell, W.R. (1977) Exp. Cell Res. 106, 261-266.
10. Stone, P.R.; Lorimer III, W.S. and Kidwell, W.R. (1977) Eur. J. Biochem. 81, 9-18.
11. Farzaneh, F. and Pearson, C.K. (1978) J. Emb. Exp. Morphol. In press.
12. Dancis, B. (1972) Personal communication.
13. Nieuwkoop, P.D. and Faber, J. (1956), Normal Table of *Xenopus laevis* (Daudin), North Holland Publishing Co., Amsterdam.