END GROUP LABELLING OF RNA AND DOUBLE STRANDED DNA BY PHOSPHATE EXCHANGE CATALYZED BY BACTERIOPHAGE T_4 INDUCED POLYNUCLEOTIDE KINASE

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SUMMARY: End group labelling of sheared double-stranded DNA, and tRNA has been effected without prior dephosphorylation, utilizing the reversal of T_4 polynucleotide kinase activity. Incubation of DNA with polynucleotide kinase in the presence and absence of a phosphate acceptor (ADP) allowed the determination of the relative ratio of 5' hydroxyl and 5' phosphoryl terminii in the polynucleotide. This method of analysis has demonstrated a high preference in the formation of 5' vs 3' phosphomonoesters during high pressure shearing of double stranded DNA.

T_2 and T_4 induced polynucleotide kinases catalyze the transfer of the γ phosphate from ATP to the 5' hydroxyl terminus of DNA, RNA and oligonucleotides (1, 2). The T_4 induced enzyme has been used extensively in the structural analysis of nucleic acids, in sequence analysis (3, 4), fingerprinting (5, 6, 7, 8) and gene synthesis (9, 10), as well as in the study of DNA enzymology (11, 12, 13, 14, 15). Since the enzyme requires a 5' hydroxyl terminus (1, 2) most DNA's, RNA's and oligonucleotides require treatment with alkaline phosphatase prior to end group labelling with polynucleotide kinase. Employing deoxyoligonucleotides as substrates, van de Sande *et al.* (16) have demonstrated the reversibility of polynucleotide kinase activity, in which the enzyme can act as an ADP dependent phosphomonoesterase. This finding suggested that the enzyme could be used to directly label 5' phosphoryl nucleic acids thereby eliminating the necessity for prior dephosphorylation. It has been shown (16) that a deoxy-5'-phosphoro-nonacosanucleotide could be quantitatively labelled by means of T_4 polynucleotide kinase catalysed phosphate exchange (no prior dephosphorylation). These findings have been extended for the end group labelling of double stranded DNA and tRNA.

Moreover, the described method permitted the direct analysis of the relative distribution of 5' and 3' phosphomonoesters formed during high pressure shearing of high molecular weight double stranded DNA.

MATERIALS AND METHODS

Enzymes. Bacteriophage T_4 induced polynucleotide kinase was purified as described previously (17). The enzyme preparation showed a single band of molecular weight ~ 35,000 daltons on analysis by SDS polyacrylamide gel electrophoresis and did not contain any detectable DNAse or RNAse activity. Bacterial alkaline phosphatase (BAPC) obtained from Worthington Biochemicals was further purified by DEAE-cellulose chromatography as described by Weiss *et al.* (11).

Polynucleotides. DNA was extracted from *E. coli* K12 by a modification of the Marmur procedure (18) and calf thymus DNA was purchased from Worthington Biochemicals. *E. coli* $tRNA^{val}$ (Boehringer) was a gift from Dr. G. H. Dixon and crude yeast tRNA (General Biochemicals) was a gift from Dr. I. Forrester. DNA sheared by passage through a needle valve at 15,000 p.s.i. displayed the same hyperchromicity as unsheared DNA. [γ-^{32}P] ATP was prepared by the method of Schendel and Wells (19) or by a modification of the procedure of Glynn and Chappell (20). The ATP was found to contain 0.4% ^{32}Pi, as analyzed by descending chromatography on DE-81 strips in 0.35 M ammonium formate. The specific activity of the [γ-^{32}P] ATP (240 Ci/mmole) was determined upon preparation by two methods developed in this laboratory (21). A DNA marker system consisting of oligomers of d-^{32}pT$(pT)_9$ was kindly provided by B. W. Kalisch (22).

Enzymatic Reactions. DNA was dephosphorylated by incubation of 35-50 nmoles of DNA nucleotide in 25 mM Tris-HCl, pH8.3 at 65°C for 45 minutes. Alkaline phosphatase (0.01 units) was added at 0, 15 and 30 minutes. Control DNA was incubated under the same conditions in the absence of the phosphomonoesterase. The alkaline phosphatase was inactivated by treatment with NaOH (23) or Nitrilotriacetic Acid (24), or the activity was inhibited by the addition of potassium phosphate to a final concentration of 4mM in the kinase reaction mixture (11). All three methods were equally effective in removing the phosphatase activity, as shown by the absence of ^{32}Pi release in the polynucleotide kinase reactions.

Phosphorylation reactions were carried out in a volume of 0.1 ml containing 50 mM Tris-HCl, pH 7.6, 2-30 nMoles of DNA or RNA nucleotide, 10 mM dithiothreitol, 5 mM $MgCl_2$, 7.5 µM [γ-^{32}P] ATP and 17.5 units of polynucleotide kinase. The exchange reactions also contained 7.5 µM ADP as a phosphate acceptor. The kinetics of the reactions were followed by the removal of 5µl aliquots and monitoring TCA precipitable counts on Whatman 3MM filters. The reactions were stopped by addition of EDTA to 25 mM followed by boiling the reaction mixture for 2 min. or by extracting the reaction mixture with chloroform:octanol (10:1).

Isolation and Characterization of ^{32}P Labelled DNA and RNA. Phosphorylated nucleic acids were separated from unreacted [γ-^{32}P] ATP by chromatography of the terminated kinase reaction mixutes on Sephadex G-200 or G-75 columns prepared in disposable 10 ml pipettes. The columns were equilibrated and eluted with 0.05 M triethylammonium bicarbonate. Fractions (0.1 ml) were collected in small glass vials and the radioactivity was determined by measuring Cerenkov radiation in a Beckman liquid scintillation counter. The fractions were concentrated by evaporation *in vacuo* and subsequently used for analysis on sucrose gradients and polyacrylamide gels.

Alkaline sucrose gradients (5-20%) were prepared in 0.1M NaOH, 0.9M NaCl and 1 mM EDTA. DNA phosphorylated using the polynucleotide kinase reaction and purified as above was mixed with identical unphosphorylated DNA as an O.D. marker and layered on the 12 ml gradients. The material was centrifuged in a Beckman SW 41 rotor at 35,000 rpm for 16 hours at 4°C. The gradients were punctured and fractions (0.3 ml) were collected by gravity flow. Radioactivity was determined by measuring Cerenkov radiation and the optical density at 260 nm was determined using a Beckman Acta III spectrophotometer.

Polyacrylamide gel electrophoresis of phosphorylated tRNA was carried out in 10% gels in the presence of 7M urea as previously described (25).

RESULTS AND DISCUSSION

In agreement with the reported T_4 polynucleotide kinase catalyzed phosphate exchange with single-stranded oligonucleotides (16), it has been possible to obtain near quantitative labelling of double-stranded DNA using this method. Figure 1 shows the kinetics of radioactive phosphate incorporation into sheared double-stranded *E. coli* DNA after incubation with polynucleotide kinase under standard conditions in the presence and absence of ADP. The reaction containing ADP displayed a six fold greater extent of labelling than the reaction lacking ADP. The difference in the extent of phosphorylation between the two reactions reflects the relative distribution of 5' hydroxyl and 5' phosphoryl terminii formed during high pressure shearing of the high molecular weight DNA. The great preference for the formation of 5' phosphoryl-terminated polynucleotides during high pressure shearing is similar to that observed for DNA fragmented by sonic irradiation (26, 27). This preference is expected due to the greater stability of the phosphate esters of primary versus secondary alcohols. Similar results were obtained using calf thymus DNA.

It was found that in the direct phosphorylation (no ADP) of sheared DNA a fast initial phosphate transfer was followed by a slow incorporation of radioactivity into DNA. This slow incorporation is probably due to the ATP catalyzed exchange reaction where ATP can act as a phosphate acceptor (16). This exchange has been minimized by decreasing the ratio of ATP to substrate. Nevertheless, the determination of the number of 5' hydroxyl groups by this method is a maximal estimate.

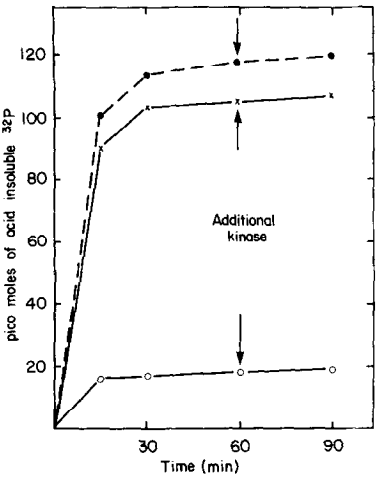

Figure 1. The kinetics of phosphorylation of sheared *E. coli* DNA. The reactions were carried out as described in Materials and Methods. The straight phosphorylation (o-o-o) contained 238 μM DNA nucleotide which had been heated at 65°/45 min. as a phosphatase control. The exchange reaction (•-•-•) contained 47.6 μM DNA nucleotide also heated at 65°/45 min. and the final reaction (x-x-x) contained 47.6 μM DNA nucleotide previously dephosphorylated as described in Materials and Methods. The kinetics were followed by removing 5 μliter aliquots and monitoring TCA precipitable counts on Whatman 3 MM filters. The reactions were started by the addition of 17.5 units of polynucleotide kinase after removal of the zero time aliquots. An additional 5.0 units of enzyme were added at 60 minutes. The data have been normalized to adjust for the differences in the amounts of DNA in the reaction mixtures.

Figure 1 also shows the conventional two step labelling procedure in which the sheared *E. coli* DNA was treated with alkaline phosphatase prior to labelling using the polynucleotide kinase. The extent of phosphorylation with the two step procedure was slightly lower than that observed with direct labelling by exchange. The difference in the extent of labelling was probably the result of incomplete dephosphorylation of the DNA by the phosphomonoesterase, leaving some 5' phosphoryl terminii which would not be labelled in the direct phosphorylation reaction. It is also noteworthy that in all polynucleotide kinase reactions using double-stranded DNA as a substrate, relatively large amounts of enzyme were required to obtain plateau levels in comparison to the amount of enzyme required to obtain quantitative label-

ling of single-stranded oligonucleotides.

Labelled DNA was isolated by gel filtration, as shown in Figure 2. This figure also shows an alkaline sucrose gradient profile of isolated sheared calf thymus DNA which was labelled by polynucleotide kinase catalyzed phosphate exchange and centrifuged with identical, unlabelled, sheared DNA. The frame shift effect observed in Figure 2 (the lack of correspondence between the optical density and radioactivity profiles) is a result of the end group labelling of fragments which have a variance in chain length. Since high pressure shearing of DNA results in the production of fragments which differ in their sizes, polynucleotide kinase catalyzed labelling results in the shorter DNA fragments being labelled to a higher specific activity per mole nucleotide than the larger fragments. Identical gradient profiles were obtained for DNA phosphorylated in the presence or absence of ADP as well as for DNA which had been treated with alkaline phosphatase prior to labelling.

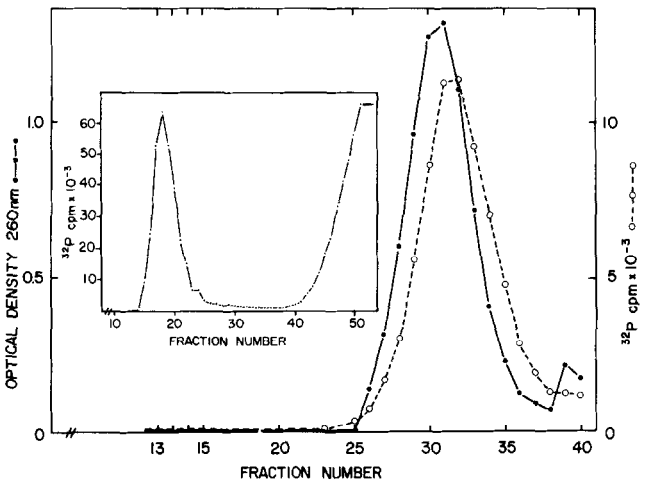

Figure 2. Alkaline sucrose gradient profile of sheared calf thymus DNA labelled by polynucleotide kinase catalyzed phosphate exchange and purified on a Sephadex G-200 column (shown in the inset). Identical unphosphorylated, sheared calf thymus DNA was included in the gradient as an O.D. marker. Details are described in Materials and Methods.

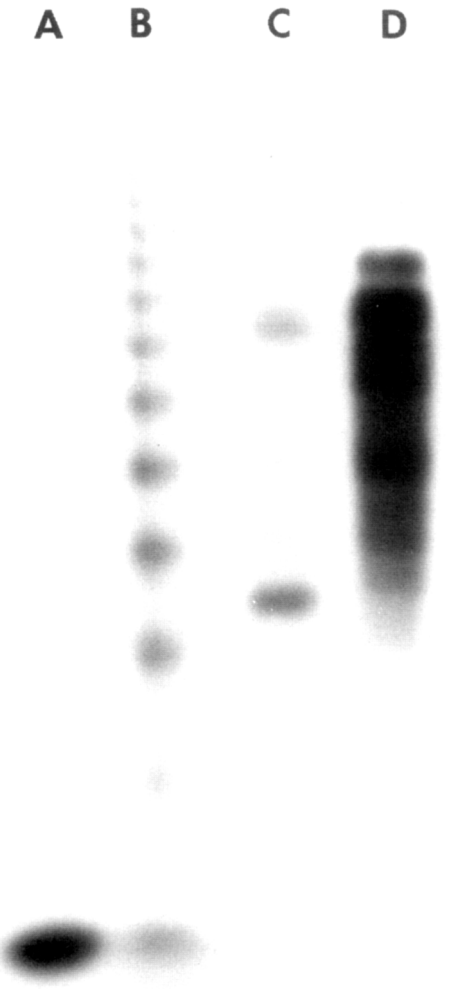

Figure 3. Autoradiograph of 10% - 7 M urea polyacrylamide gel (20X30 cm) of $^{32}pT(pT)_9$ (slot A) along with a marker system of $[^{32}pT(pT)_9]_n$ (where n=1, 2, 3, 4....) (slot B) and phosphorylated *E. coli* $tRNA^{val}$ (slot C) and yeast tRNA (slot D). The tRNA's were labelled by polynucleotide kinase catalyzed phosphate exchange and purified from unreacted $[\gamma\text{-}^{32}P]ATP$ as described in Materials and Methods. The gel was run at room temperature for 20 hours at 200 mV.

The polynucleotide kinase catalyzed phosphate exchange reaction has also been used to label crude yeast tRNA and *E. coli* $tRNA^{val}$. The kinetics of phosphorylation of these RNA's reached plateau levels which could not be increased upon further addition of enzyme. The labelled RNA preparations were purified from unreacted [γ-^{32}P]ATP by gel filtration and analyzed by electrophoresis in a 10%-7 M urea polyacrylamide gel (Figure 3). A DNA marker system consisting of polymers of d-^{32}pT(pT)$_9$ was run on the same gel. Under denaturing conditions the labelled tRNA's migrated in the expected manner in relation to the oligothymidilate markers. The analysis by gel electrophoresis showed that both RNA preparations contained small RNA fragments which because of their size were labelled to a high specific radioactivity per mole nucleotide and therefore exhibited bands on the autoradiogram which were stronger than would be expected from their molar concentration.

In conclusion, the reversal of T_4 polynucleotide kinase activity can be used to directly label 5' phosphoryl-terminated double-stranded nucleic acids without prior dephosphorylation. The use of double-stranded nucleic acids necessitates extremely large quantities of enzyme to reach plateau levels of phosphorylation, and high pressure shearing of DNA results in the preferential formation of 5' phosphomonoesters. It should also be emphasized that low level phosphate exchange may occur in the absence of ADP and may complicate experiments designed to label only 5' hydroxyl terminii. Okazaki *et al.* (28) have recently reported the elimination of low levels of exchange by running polynucleotide kinase reactions at 0°C. It is also possible to minimize the reversal reaction by working at a higher pH (the optimum pH of the forward reaction is 9.5 while that of the reverse is 6.5) or by using a low ATP to substrate ratio (16, 28, unpublished results).

The end group labelling of sheared DNA by the methods described above has resulted in the development of a reliable and easily prepared DNA probe of high specific radioactivity whose use in DNA reassociation and RNA-DNA hybridization experiments is currently under investigation.

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REFERENCES

1. Novogrodsky, A. and Hurwitz, J. (1966) *J. Biol. Chem. 241,* 2923.
2. Richardson, C.C. (1965) *Proc. Nat. Acad. Sci. U.S. 54,* 158.
3. Weiss, B. and Richardson, C.C. (1967) *J. Mol. Biol. 23,* 405.
4. Jay, E., Bambara, R., Padmanabhan, R. and Wu, R. (1974) *Nucleic Acid Res. 1,* 331.
5. Székely, M. and Sanger, F. (1969) *J. Mol. Biol. 43,* 607.
6. Southern, E.M. (1970) *Nature (London) 227,* 794.
7. Murray, K. (1973) *Biochem. J. 131,* 569.
8. Simsek, M., Ziegenmeyer, J., Heckman, J., and RajBhandary, U.L. (1973) *Proc. Nat. Acad. Sci. U.S. 70,* 1041.
9. Agarwal, K.L., Büchi, H., Caruthers, M.H., Gupta, N., Kleppe, K., Kumar, A., Ohtsuka, E., RajBhandary, U.L., van de Sande, J.H., Sgaramella, V., Weber, H., Yamada, T. and Khorana, H.G. (1970) *Nature 227,* 27.
10. Caruthers, M.H., Agarwal, K., Besmer, P., Cashion, P., Fridkin, M., Jay, E., Kumar, A., Loewen, P., Miller, R., Minamoto, K., Rama Moorty, B., Panet, A., van de Sande, J.H., Sekia, T., Siderova, N., Khorana, H.G. (1973) Ninth International Congress of Biochemistry, Stockholm, Abs. 3010, p. 194.
11. Weiss, B., Live, T.R., and Richardson, C.C. (1968) *J. Biol. Chem. 243,* 4530.
12. Gefter, M.L., Becker, A., and Hurwitz, J. (1967) *Proc. Nat. Acad. Sci. U.S. 58,* 1723.
13. Olivera, B.M. and Lehman, G.R. (1967) *Proc. Nat. Acad. Sci. U.S. 57,* 1426.
14. Becker, A. and Hurwitz, J. (1967) *J. Biol. Chem. 242,* 3685.
15. Weiss, B., Jacquemin-Sablon, A., Live, T.R., Fareed, G.C. and Richardson, C.C. (1968) *J. Biol. Chem. 243,* 4543.
16. van de Sande, J.H., Kleppe, K. and Khorana, H.G. (1973) *Biochemistry 12,* 5050.
17. Panet, A., van de Sande, J.H., Loewen, P.C., Khorana, H.G., Raae, A.J. Lillehaug, J.R. and Kleppe, K. (1973) *Biochemistry 12,* 5045.
18. Marmur, J. (1961) *J. Mol. Biol. 3,* 208.
19. Schendel, P.F. and Wells, R.D. (1973) *J. Biol. Chem. 248,* 8319.
20. Glynn, G.M. and Chappell, J.B. (1964) *Biochemical J. 90,* 147.
21. Chaconas, G., van de Sande, J.H. and Church, R.B. *Anal. Biochem., in press.*
22. Kalisch, B.W. and van de Sande, J.H. Manuscript in preparation.
23. Jacquemin-Sablon, A. and Richardson, C.C. (1970) *J. Mol. Biol. 47,* 477.
24. Simsek, M., Ziegenmeyer, J., Heckman, J. and RajBhandary, U. L. (1973) *Proc. Nat. Acad. Sci. U.S. 70,* 1041.
25. Maniatis, T., Jeffrey, A., and van de Sande, J.H. *Biochemistry, in press.*
26. Richards, O.C. and Boyer, P.D. (1965) *J. Mol. Biol. 11,* 327.
27. Richardson, C.C. (1966) *J. Mol. Biol. 15,* 49.
28. Okazaki, R., Hirose, S., Okazaki, T., Ogawa, T. and Kurosawa, Y. (1975) *Biochem. Biophys. Res. Comm. 62,* 1018.