RNA 3'-terminal phosphate cyclase activity and RNA ligation in HeLa cell extract

W.Filipowicz*+, M.Konarska*, H.J.Gross§ and A.J.Shatkin+

*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warsaw, Poland, §Institut für Biochemie, Universität Würzburg, D-8700 Würzburg, FRG and +Roche Institute of Molecular Biology, Nutley, NJ 07110, USA

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ABSTRACT

HeLa cell extract contains RNA ligase activity that converts linear polyribonucleotides to covalently closed circles. RNA substrates containing 2',3'-cyclic phosphate and 5'-hydroxyl termini are circularized by formation of a normal 3',5' phosphodiester bond. This activity differs from a previously described wheat germ RNA ligase which circularizes molecules with 2',3'-cyclic and 5' phosphate ends by a 2'-phosphomonoester, 3',5'-phosphodiester linkage (Konarska et al., Nature 293, 112-116, 1981; Proc. Natl. Acad. Sci. USA 79, 1474-1478, 1982). The HeLa cell ligase can also utilize molecules with 3'-phosphate ends. However, in this case ligation is preceded by an ATP-dependent conversion of the 3'-terminal phosphate to the 2',3' cyclic form by a novel activity, RNA 3'-terminal phosphate cyclase. Both RNA ligase and RNA 3'-terminal phosphate cyclase activities are also present in extract of Xenopus oocyte nuclei, consistent with a role in RNA processing.

INTRODUCTION

Transcripts of many eukaryotic genes contain intervening sequences (introns) which are removed by excision-ligation during RNA splicing (1,2). In some cases the excised sequences have been detected as covalently closed circular molecules (3-5). Other naturally occurring circular RNAs include viroids (6) and viroid-like RNAs in plant viruses (7). The requirement for a ligation step during maturation of many different types of RNA has raised considerable interest in eukaryotic RNA ligases, although it was shown recently that a precursor of Tetrahymena ribosomal RNA can undergo excision-ligation in the absence of protein (8).

Enzymes that catalyse the joining of tRNA half molecules in extracts of yeast and X. laevis nuclei (9,10) were the first eukaryotic RNA ligases to be reported. Previously we characterized an RNA ligase activity in wheat germ extract by measuring the intramolecular ligation (circularization) of a model RNA substrate, called Ω. In this reaction, ligation was dependent on the presence of 3'-terminal 2',3'-cyclic phosphate and 5'-phosphate ends, although 5'-hydroxylated Ω was also circularized due to 5'-phosphorylation by poly-

nucleotide kinase activity in the extract. The two ends of Ω were joined by an unusual 2'-phosphomonoester, 3',5'-phosphodiester linkage, $N\overset{p}{<}_{p}N$, in which the 2'-phosphate derives from the cyclophosphate end and the phosphodiester bond from the 5' phosphate (11,12). Recently it was shown that the $N\overset{p}{<}_{p}N$ linkage is formed during ligation of tRNA half molecules in systems derived from yeast, wheat germ and Chlamydomonas reinhardtii (Abelson et al.; K. Tyc, Y. Kikuchi, M.K., W.F. and H.J.G., manuscripts submitted). Natural and RNase T1-nicked linear viroids are also circularized by wheat germ ligase (13), and in both RNAs ligation occurs via $N\overset{p}{<}_{p}N$ linkage (14).

In the present study we report on the presence in HeLa cell extract of another type of RNA ligase. The HeLa enzyme uses a 2,3'-cyclic phosphate for ligation. However, in contrast to the plant activities, a normal 3',5'-phosphodiester linkage is formed by derivation of the joining phosphate from the 3'-terminal cyclic phosphate. Consistent with utilization of a cyclic phosphate terminus for RNA ligation, HeLa cell extract contains an ATP-dependent activity that converts RNA 3'-terminal phosphate to the 2',3'-cyclic form.

MATERIALS AND METHODS

Preparation of Substrates for Ligase Assay

RNA from the SPS isolate of tobacco mosaic virus was used to prepare the RNase T1-resistant, 73-nucleotide Ω oligonucleotide (15). Ω 3'-end-labeled with [5'-^{32}P]pCp ($\Omega\overset{*}{p}Cp$) and Ω containing 3'-terminal [^{32}P]G$^{3'}$p or [^{32}P]G>p (referred to as $\Omega^{3'*}p$ and $\Omega{>}\overset{*}{p}$, respectively) were derived as described (12). 5'[^{32}P]-phosphorylated Ω enriched in molecules containing 3'-terminal 2',3'-cyclic phosphate ($\overset{*}{p}\Omega{>}p$) was prepared as described (12) with T4 pseT 1 mutant polynucleotide kinase (devoid of 3'-phosphatase activity, New England Nuclear Corp.).

[^{32}P]G$^{2'}$p-terminated Ω ($\Omega^{2'*}p$) was derived from $\Omega{>}\overset{*}{p}$ by treatment with calf brain 2',3'-cyclic nucleotide 3'-phosphohydrolase (kindly provided by Drs. J. Hofsteenge and S. Moore, Rockefeller University, New York) as follows. $\Omega{>}\overset{*}{p}$ (1 x 10^5 cpm) was incubated for 45 min at 30°C with 0.1 unit (2',3'-cyclic NADP units, ref. 16) of the brain enzyme in 10 ul of 0.2 M 2-[N-morpholino]-ethanesulfonic acid, pH 6.0 containing 5% glycerol, and the resulting $\Omega^{2'*}p$ was purified by polyacrylamide gel electrophoresis. Analysis of the 3' terminus indicated that the conversion of $\Omega{>}\overset{*}{p}$ to $\Omega^{2'*}p$ was complete (see Fig. 5E). Ω

labeled with [5'-^{32}P]pdCp at the 3'-terminus (Ω *pdCp) was prepared as described for Ω^{*}pCp (12) except that [5'-^{32}P]pdCp, made by 5'-phosphorylation of deoxycytidine 3'-monophosphate in the presence of T4 polynucleotide kinase and [γ-^{32}P]ATP (17), was used in place of [5'-^{32}P]pCp. [γ-^{32}P]ATP and [5'-^{32}P]pCp (each ~2,000 Ci/mmol) were obtained from Amersham.

Cell Extracts

A cell-free S-100 extract was prepared from HeLa cells as described by Weil et al. (18) and dialysed (3 x 5 hr, 100 vol. each) against 40 mM Hepes-KOH, pH 7.9 containing 120 mM KCl, 4 mM Mg acetate, 0.5 mM dithiothreitol (DTT), and 10% glycerol (vol/vol).

Nuclei (germinal vesicles) were isolated manually from Xenopus laevis oocytes, and extract was prepared according to Birkenmeier et al. (19). Germinal vesicles were washed and suspended (1 vesicle/ul) in Buffer J (19) before disruption by passage 10 times through a plastic pipette tip. Lysate was centrifuged for 5 min at 10,000 rpm and stored frozen at -140°C.

Ligation Assays

Ω substrates were incubated in 10 ul reactions containing (unless otherwise indicated) 20 mM Hepes-pH 7.9, 90 mM KCl, 10 mM Mg acetate, 1 mM DTT, 0.1 mM EDTA, 25 mM sodium phosphate, 80 uM spermine, 1 mM ATP, 4% glycerol, 1 ul of HeLa cell extract (protein concentration 9-11 mg/ml) and 2-5 x 10^3 cpm of Ω substrate. Incubations were for 5 min at 20°C unless specified otherwise.

Germinal vesicle extract was tested for RNA ligase activity under the same conditions except that 10 ul incubation mixtures contained 3 ul of extract and also 20 mM NH_4Cl; incubations were for 15 min at 25°C. Reactions containing HeLa cell or germinal vesicle extract were terminated by addition of $NaDodSO_4$ (0.1% final) and 10 ug of proteinase K (Merck). Samples were incubated for 30 min at 37°C, supplemented with urea and subjected to electrophoresis in 10% polyacrylamide-8 M urea gels (15). RNA ligations in wheat germ extract were as described previously, and incubation mixtures were applied directly to gels (12). RNAs were isolated from gels in the presence of 5 ug of carrier tRNA and subjected to digestion by nuclease, phosphatase or alkali as described (11,12). Digests were analysed by thin-layer chromatography on cellulose plates in solvent A [saturated $(NH_4)_2SO_4$/1 M Na acetate/isopropanol, 80:18:2 (vol/vol)] or solvent B [isobutyric acid/NH_4OH/H_2O, pH 4.3, 577:38:385 (vol/vol)]. Autoradiography was at -70°C using Kodak X-Omat AR film and DuPont Cronex Lightning-Plus screens.

RESULTS

HeLa Cell Extract Contains RNA Ligase that Joins RNA Ends via Normal 3',5' Phosphodiester Linkage

HeLa cell extract was assayed for RNA ligase activity by measuring circularization of Ω substrates bearing different termini. Figure 1A indicates that incubation of Ω>$\overset{*}{p}$ (lane 3) and Ω$^{3'*}$p (lane 5) leads to the formation of products that migrate in polyacrylamide gels in the position of marker Ω circles (Ω_c) formed either in wheat germ extract (lane 1) or by phage T4 RNA ligase (Fig. 1B, lane 1). $\overset{*}{p}$Ω>p, an active ligase substrate in the wheat germ system (12), was not circularized by HeLa cell extract (Fig. 1A, lane 7); molecules bearing 5' phosphate and 3' hydroxyl termini also were not ligated (data not shown).

The slowly migrating radiolabeled molecules generated in HeLa extract from Ω>$\overset{*}{p}$ and Ω$^{3'*}$p were recovered from the gel and subjected to further analysis. Mild alkali treatment of both preparations (Fig. 1B and data not shown) yielded degradation patterns characteristic of single-nick conversion of circular RNA to its linear counterpart (12,15). The nature of the intramolecular end-to-end linkage in both Ω_c preparations was investigated by digestion with nuclease P1 and RNase T2 followed by thin layer chromatography of the resulting nucleotides. The Ω_c products each yielded $\overset{*}{p}$U and G$\overset{*}{p}$ following treatment with nuclease P1 and RNase T2 respectively (Fig. 1C, lanes 3-6). The identity of the nucleotides was confirmed by chromatographic analysis in another solvent system consisting of t-butanol/HCl/H_2O, 14:3:3 (data not shown). These results establish the structure of the linkage in Ω_c in each case as a normal 3',5'-phosphodiester, G$\overset{*}{p}$U (Ω terminal sequences are 5'-UA... 69N... UG-3', ref. 15). In contrast, Ω>$\overset{*}{p}$ circularization in wheat germ extract yields the 2'-phosphomonoester, 3',5'-phosphodiester linkage, G$\overset{p}{\underset{p}{<}}$U (Fig. 1C, lanes 1 and 2; ref. 12). Circularization of Ω>$\overset{*}{p}$ or Ω$^{3'*}$p was prevented by pre-incubation of the HeLa cell extract for 10 min at 55°C or with 1 mg/ml proteinase K (30 min, 20°C), indicating that the ligation reaction is protein-dependent. In addition, the extent of substrate circularization was proportional to protein concentration in the range 3-15 ug/assay (data not shown).

HeLa Cell Extract Contains RNA 3'-terminal Phosphate Cyclase Activity

The kinetics of circularization of Ω>$\overset{*}{p}$ and Ω$^{3'*}$p in HeLa cell extract were compared. A maximum level of Ω>$\overset{*}{p}$ circularization occurs within 1 min at 20°C (Fig. 2). Time-dependent Ω>$\overset{*}{p}$ ligation can be demonstrated at 0 or 5°C; at these temperatures plateau values of 36-41% Ω_c were reached in 3-5 min

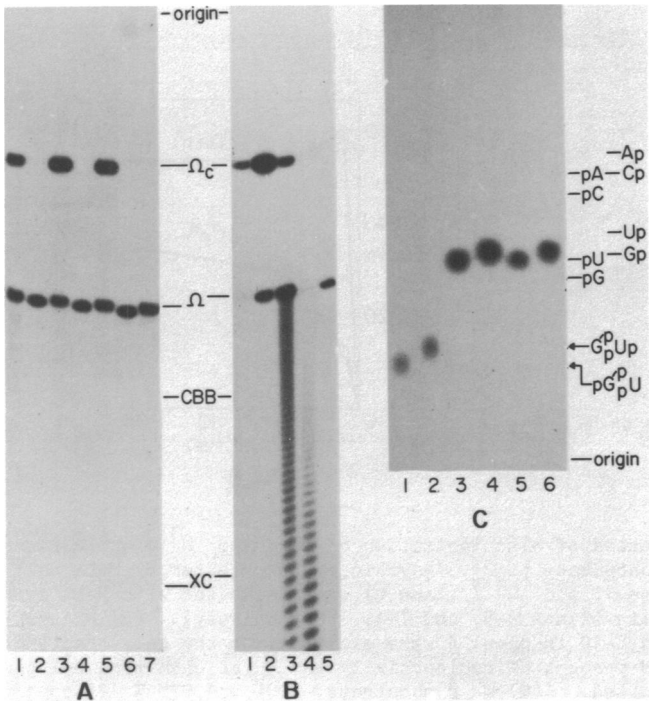

Fig. 1. Conversion of linear Ω oligonucleotides to covalently closed circular molecules in HeLa cell extract. (A) Ω molecules containing different terminal structures were incubated with HeLa cell or wheat germ (w.g.) extract, and the products were analysed by polyacrylamide gel electrophoresis. (lane 1) Ω>p, w.g.; (2) Ω>p; (3) Ω>p, HeLa; (4) Ω3'p; (5) Ω3'p, HeLa; (6) p Ω>p; (7) p Ω>p, HeLa. The positions of linear (Ω) and circular (Ω_c) forms of the oligonucleotide and of Coomassie brilliant blue (CBB) and Xylene cyanol (XC) dyes are indicated. Incubations with HeLa cell extract were for 15 min and mixtures contained 3 µl of the extract. (B) Mild alkaline treatment of Ω_c product formed from Ω>p by incubation with HeLa cell extract. Ω_c from panel A, lane 3, was isolated and treated with 50 mM NaOH at 80°C for 0, 1 and 3 min (lanes 2-4, respectively); lane 1, Ω_c formed by T4 RNA ligase (ref. 15); lane 5, Ω>p. (C) Products of enzymatic digestion of Ω_c. Ω_c generated from Ω>p by incubation with wheat germ (lanes 1,2) or HeLa extract (lanes 3,4) was digested with nuclease P1 (lanes 1,3) and RNase T2 (lanes 2,4). Lanes 5,6 are the same as lanes 3,4 except that Ω_c was from Ω3'p. Cellulose plates were developed in solvent B. Mononucleotide markers were as indicated, and arrows show expected positions of 2'-phosphate-containing dinucleotides (11,12).

(data not shown). Ligation of Ω3'p at 20°C proceeds considerably more slowly than Ω>p circularization, and the reaction continues for 5 min or longer (Fig. 2A and B). The results suggested that Ω3'p can be converted to Ω>p in HeLa cell extract. To test this possibility, molecules that remained linear after

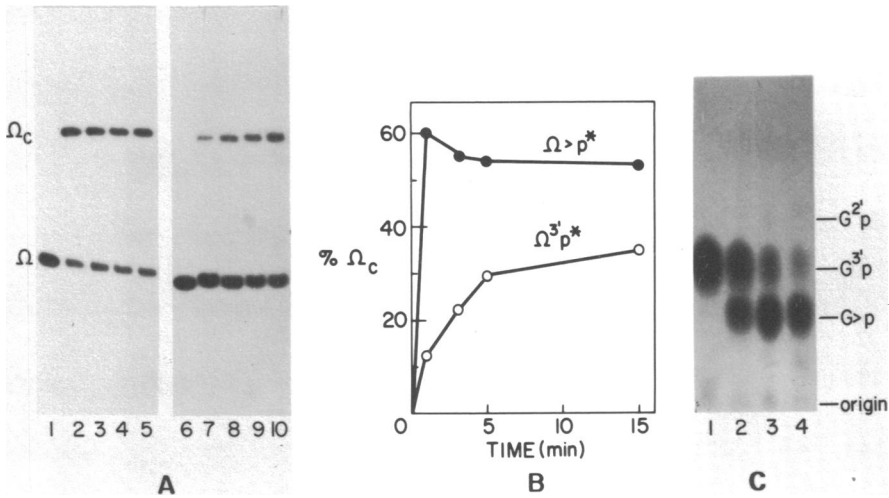

Fig. 2. Kinetics of circularization of $\Omega{>}p^{*}$ and $\Omega^{3'*}p$ and conversion of the 3'-terminal phosphate to 2',3'-cyclic phosphodiester by HeLa cell extract. (A) $\Omega{>}p^{*}$ (lane 1) and $\Omega^{3'*}p$ (lane 6) were incubated with HeLa extract for 1, 3, 5 and 15 min (lanes 2-5, and 7-10, respectively). (B) Ω and Ω_c from lanes 2-5 and 7-10 in panel A were excised from the gel; radioactivity was measured, and percent of radioactivity in Ω_c at each time point was calculated and plotted. (●) $\Omega{>}p^{*}$ substrate, 100% = 1,025 ± 125 cpm; (○) $\Omega^{3'*}p$ substrate, 100% = 1,170 ± 130 cpm. (C) Analysis of 3' termini of material in the position of linear Ω after incubation of $\Omega^{3'*}p$ with HeLa extract (panel A, lanes 6-10). Ω was recovered from the gel, digested with RNase A and analysed by thin-layer chromatography in solvent A. Lane 1, control $\Omega^{3'}p$; (2-4) linear material after incubation with HeLa cell extract for 1, 5, and 15 min, respectively.

incubation with HeLa cell extract (Fig. 2A) were analysed by RNase A digestion and chromatography. Material that corresponded to pure $\Omega{>}p^{*}$ before incubation yielded $G{>}p^{*}$, demonstrating retention of the cyclic phosphate 3'-termini (data not shown). However, the digestion products originating from $\Omega^{3'*}p$ indicated that there was a time-dependent decrease in the amount of 3'-terminal $G^{3'*}p$ and a concomitant accumulation of a spot that comigrated with $G{>}p^{*}$ (Fig. 2C), consistent with conversion of $\Omega^{3'*}p$ to $\Omega{>}p^{*}$ in HeLa cell extract. Figure 3 shows that circularization of $\Omega^{3'*}p$ (panel A) and formation of 3'-terminal cyclic phosphate (panel B) both require ATP. The α-β- and β-γ-methylene analogs do not substitute for ATP, suggesting a requirement for hydrolysis of both pyrophosphate linkages or that the analogs are not recognized by the cyclase.

To confirm that $\Omega^{3'*}p$ is converted to $\Omega{>}p^{*}$ in HeLa cell extract, linear Ω

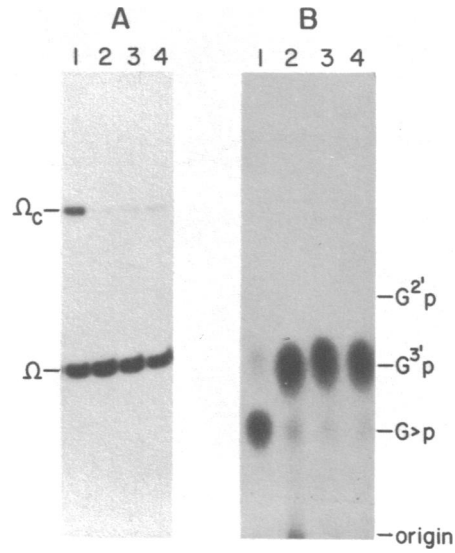

Fig. 3. ATP hydrolysis requirement for circularization of $\Omega^{3'*}p$ (A) and generation of 2,3'-cyclic phosphate termini (B). (A) $\Omega^{3'*}p$ was incubated in the presence of 1 mM ATP (lane 1) or $AMP(CH_2)PP$ (lane 2) and $AMPP(CH_2)P$ (lane 3) in place of ATP; (lane 4) ATP omitted. (B) Radioactive material in the position of linear Ω in panel A was recovered from the gel, treated with RNase A and subjected to thin-layer chromatography in solvent A. (Lanes 1,2) incubation with and without ATP, respectively; (Lanes 3,4) $AMP(CH_2)PP$ and $AMPP(CH_2)P$ in place of ATP, respectively. Analogs were purchased from Miles.

molecules recovered from a gel after incubation in the extract were treated with different enzymes and the products analyzed. Digestion of control non-incubated $\Omega^{3'*}p$ with nuclease P1 released from 3'-termini P_i as the only radioactive product, due to the 3'-phosphatase activity of this enzyme (Fig. 4, lanes 1 and 6). P1 nuclease digestion of extract-treated Ω yielded P_i and material which co-migrated with authentic pG>p in two different solvent systems (lanes 2 and 7). Treatment of control $\Omega^{3'*}p$ with calf intestine phosphatase released P_i (lane 4), while the extract-treated Ω material yielded half of the radioactivity as material that remained at the origin (lane 5), consistent with conversion of $\Omega^{3'*}p$ to phosphatase-resistant $\Omega>p^*$. The presumptive $pG>p^*$ from lane 2 was isolated and characterized further. As expected, it was resistant to digestion by nuclease P1 (lane 9) but converted quantitatively to $pG^{3'*}p$ by RNase T1 or T2 (lanes 10 and 11) and to a mixture of $pG^{3'*}p$ and $pG^{2'*}p$ by exposure to alkali (lane 13). $pG^{3'*}p$ generated by treating $pG>p^*$ with RNase T2 or alkali was completely sensitive to nuclease P1 (lanes 12 and 14) while $pG^{2'*}p$ was

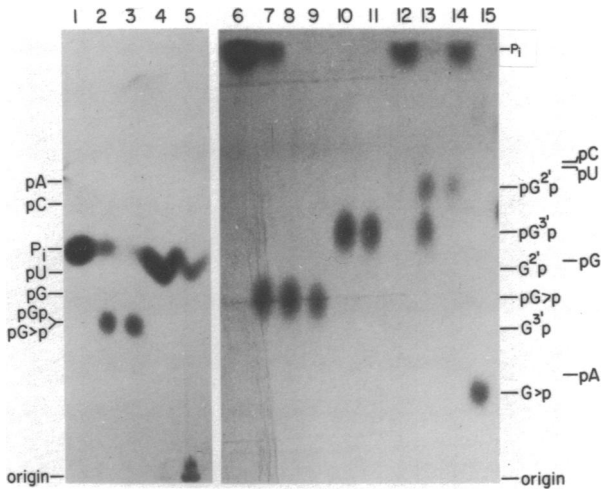

Fig. 4. Conversion of $\Omega^{3'*}p$ to $\Omega>p^{*}$ in HeLa extract: analyses of 3'-terminal structures. Nuclease P1 digests of $\Omega^{3'*}p$ (lanes 1 and 6); linear Ω material recovered from a gel following incubation of $\Omega^{3'*}p$ with HeLa extract (lanes 2,7); and $\Omega>p^{*}$ (lanes 3,8). Lanes 4 and 5 show calf intestine phosphatase digests of $\Omega^{3'*}p$ and linear Ω recovered after incubation with HeLa extract, respectively. For lanes 9-15, presumptive $pG>p^{*}$ from lane 2 was isolated and subjected to treatment with: nuclease P1 (9); RNase T1 (10); RNase T2 (11); RNase T2 followed by nuclease P1 (12); NaOH (13); NaOH followed by nuclease P1 (14); calf intestine phosphatase (15). Chromatography was in solvent B (lanes 1-5) or solvent A (6-15).

largely resistant (lane 14). Digestion of $pG>p^{*}$ with calf intestine phosphatase yielded $G>p^{*}$ (lane 15). These results support the conclusion that HeLa cell extract contains RNA 3'-terminal phosphate cyclase that converts $\Omega^{3'*}p$ to $\Omega>p^{*}$.

The presence in HeLa cell extract of RNA 3'-terminal cyclase is further supported by the results shown in Fig. 5. $\Omega^{*}pCp$ was circularized by HeLa cell extract (Fig. 5A, lane 2). Circular and linear forms of Ω were isolated from lane 2 and treated with nuclease P1; Ω_c yielded pC^{*} (Fig. 5B, lane 1), while treatment of linear Ω yielded a radioactive spot comigrating with $pC^{3'}p$ marker (Fig. 5B, lane 2), which is also the position of pC>p in this system. Identification of this spot as $pC>p^{*}$ was based on the following properties. It was resistant to nuclease P1 digestion (Fig. 5C, lane 1; untreated material migrated in the same position). By RNase T2 digestion it yielded $pC^{3'*}p$ (Fig. 5C, lane 2) which was converted to pC^{*} by nuclease P1 (lane 3). Treatment with NaOH produced $pC^{2'*}p$ and $pC^{3'*}p$ (lane 4), and only the latter nucleotide yielded pC^{*} when alkali treatment was followed by nuclease P1 digestion (lane 5).

Independent demonstration that $\Omega^{*}pCp$ is converted to $\Omega pC>p^{*}$ in HeLa cell

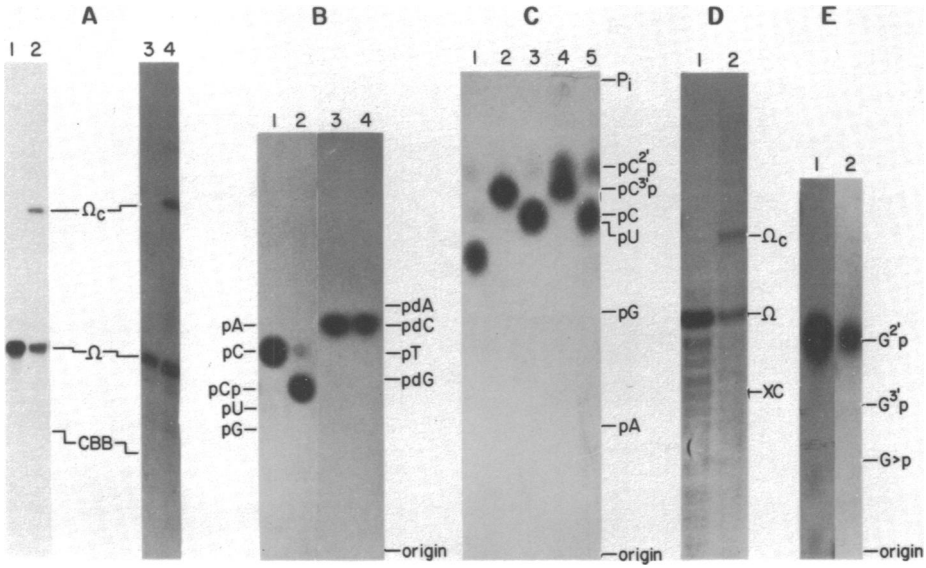

Fig. 5. Comparison of $\Omega\overset{*}{p}Cp$, $\Omega\overset{*}{p}dCp$ and $\Omega^{2'}\overset{*}{p}$ as substrates for RNA 3'-terminal phosphate cyclase and RNA ligase activities in HeLa cell extract. (A) Gel electrophoresis of ligase assays containing as substrates: $\Omega\overset{*}{p}dCp$ (lane 1); $\Omega\overset{*}{p}Cp$ (2); $\Omega^{2'}\overset{*}{p}$ (3); or $\Omega^{3'}\overset{*}{p}$ (4). Incubations with HeLa cell extract were for 15 min and contained 3 µl of extract. (B) Analysis of nuclease P1 digests of Ω_c derived from $\Omega\overset{*}{p}Cp$ (lane 1); linear material recovered from the gel after incubation of $\Omega\overset{*}{p}Cp$ (2) and $\Omega\overset{*}{p}dCp$ (4) with HeLa extract; lane 3, $\Omega\overset{*}{p}dCp$ before incubation. Nuclease P1 digestion of control non-incubated $\Omega\overset{*}{p}Cp$ yielded $\overset{*}{p}C$. Chromatography in solvent B. (C) Presumptive $\overset{*}{p}C>p$ from track 2 in panel B was isolated and treated with nuclease P1 (lane 1); RNase T2 (2); RNase T2 followed by nuclease P1 (3); NaOH (4); NaOH followed by nuclease P1 (5). Chromatography in solvent A. (D) Wheat germ ligase-catalysed circularization of $\Omega\overset{*}{p}Cp$ (lane 1) and linear Ω recovered from the gel after incubation of $\Omega\overset{*}{p}Cp$ with HeLa extract (2). Electrophoresis was in a 12% polyacrylamide gel. (E) Analysis of the 3'-termini of $\Omega^{2'}\overset{*}{p}$ before (track 1) and after (track 2) incubation with HeLa cell extract. Ω molecules were digested with RNase A and digests were chromatographed in solvent A.

extract is shown in Fig. 5D. $\Omega\overset{*}{p}Cp$ was incubated in HeLa cell extract and the linear Ω recovered from a gel was re-incubated in wheat germ extract. The plant RNA ligase activity circularizes only Ω molecules containing preformed 2',3'-cyclic phosphate termini (12). Accordingly, control $\Omega\overset{*}{p}Cp$ that had not been exposed to HeLa cell extract was not circularized by wheat germ extract (Fig. 5D, lane 1). In contrast, linear Ω that had been pre-incubated in HeLa cell extract was ligated (lane 2).

Two other Ω molecules, the 2'-deoxy-terminated $\Omega\overset{*}{p}dCp$ and $\Omega^{2'}\overset{*}{p}$ were tested as potential RNA ligase and 3' phosphate cyclase substrates in the HeLa

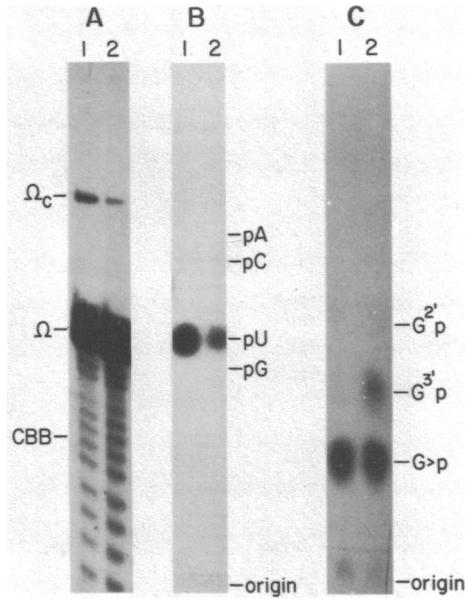

Fig. 6. Incubation of $\Omega>p^{*}$ and $\Omega^{3'*}p$ with germinal vesicle extract and analysis of reaction products. (A) Gel electrophoresis of ligation reactions containing $\Omega>p^{*}$ (lane 1) and $\Omega^{3'*}p$ (lane 2). (B) Nuclease P1 digestion products of Ω_c from $\Omega>p^{*}$ (track 1) and $\Omega^{3'*}p$ (track 2). Chromatography in solvent B. (C) RNase A digestion products of linear Ω recovered after incubation of $\Omega>p^{*}$ (track 1) and $\Omega^{3'*}p$ (track 2) in extract. Chromatography in solvent A.

cell extract. Neither was circularized (Fig. 5A, lanes 1 and 3). Furthermore, $\Omega^{*}pdCp$ both before and after incubation with HeLa cell extract yielded $^{*}pdC$ by nuclease P1 digestion, as determined by chromatography in solvent B (Fig. 5B, lanes 3 and 4) and solvent A (data not shown). RNase A digestion of $\Omega^{2'*}p$ released $G^{2'*}p$ indicating that no cyclization of the 2'-phosphate occurred in HeLa cell extract (Fig. 5E). The findings demonstrate that $\Omega^{*}pdCp$ and $\Omega^{2'*}p$, molecules that cannot form a 2',3' cyclic phosphate terminus, also are not ligation substrates.

Extracts of Xenopus Oocyte Nuclei Contain RNA 3'-phosphate Cyclase and Ligase Activities

If the RNA 3'-phosphate cyclase and ligase activities play a role in RNA maturation one would predict their presence in nuclei. Germinal vesicles (nuclei) were manually dissected from Xenopus laevis oocytes to avoid cytoplasmic contaminants, and extract was prepared. $\Omega>p^{*}$ and $\Omega^{3'*}p$ were both circu-

larised in nuclear extract (Fig. 6A), but the yields of Ω_c were lower than in HeLa cell extract. Low ligase activity was also found in HeLa whole cell extract that includes the contents of nuclei (i.e. extract prepared according to Manley et al., ref. 20). In addition, HeLa whole cell extract strongly inhibited RNA ligation when mixed with the HeLa cell S100 extract used in this work (data not shown). These findings may account for the relatively weak RNA ligase activity in the Xenopus nuclear extract.

Circular material obtained by incubation of $\Omega{>}p^*$ and $\Omega^{3'*}p$ in Xenopus nuclear extract was examined by nuclease P1 digestion and chromatography. In each case pU^* was the only radioactive product (Fig. 6B). The results show that, as in the case of the HeLa cell extract, Ω ligation in the Xenopus nuclear extract is via formation of a Gp^*U 3',5'-phosphodiester linkage. Linear Ω was also recovered from the gel and analysed after treatment with RNAse A. $G{>}p^*$ was obtained from Ω molecules which, prior to incubation in nuclear extracts, corresponded to $\Omega{>}p^*$ (Fig. 6C, lane 1). Linear Ω derived from $\Omega^{3'*}p$ also yielded mainly $G{>}p^*$ and low amounts of $G^{3'*}p$ (Fig. 6C, lane 2). Thus Xenopus germinal vesicle extract contains RNA 3'-terminal phosphate cyclase activity that catalyzes the conversion of $\Omega^{3'}p$ to $\Omega{>}p$.

DISCUSSION

Two novel activities have been identified in HeLa cell extracts. They include 3'-terminal phosphate cyclase and RNA ligase which uses 2',3'-cyclic phosphate termini and joins RNA ends via 3',5'-phosphodiester linkage. These two activities are also present in extracts of Xenopus oocyte germinal vesicles, suggesting that they may be involved in RNA processing in the nucleus. The cyclase appears to have little sequence or terminal nucleotide specificity. In addition to $\Omega^{3'}p$ and ΩpCp, HeLa cell extract catalyzed cyclization of the 3'-phosphate of [^{32}P]pCp-labeled E. coli 5S RNA and $tRNA_f^{Met}$, yeast $tRNA^{Phe}$ and $oligo(A)_{16-22}$; $pC^{3'}p$ mononucleotide was not a substrate (unpublished results). The cyclizing activity was heat sensitive (65°, 10 min), and the extent of $\Omega^{3'}p$ conversion to $\Omega{>}p$ was dependent on incubation time and cell extract concentration (Fig. 2C, data not shown). However, treatment of cell extract or partially purified material with trypsin, chymotrypsin, proteinase K or pronase did not destroy the activity.

Conversion of $\Omega^{3'}p$ to $\Omega{>}p$ requires ATP, and the α-β and β-γ-methylene analogs are inactive. The mechanism of ATP action in the cyclization reaction is unknown. However, by analogy with T4 RNA ligase which activates the 5' phosphate of donor molecules by $A^{5'}pp^{5'}N$ formation (21), α-β bond cleavage may

be necessary for adenylylation of the 3'-terminal phosphate. Formation of the activated structure, $N^{3'}pp^{5'}A$ would yield a 2',3'-cyclic phosphate terminus upon subsequent elimination of AMP, a good leaving group in this reaction. In fact, Hinton et al. (22) have observed that in the absence of its natural, 5'-phosphorylated substrate, T4 RNA ligase can activate 3'-phosphate terminated oligodeoxyribonucleotides at a low rate. In this reaction AMP is added to the 3' phosphate to form a $dN^{3'}pp^{5'}A$ structure. Similar reaction of T4 RNA ligase with 3'-phosphate terminated oligoribonucleotides yielded low amounts of products with 2',3'-cyclic phosphate ends. By using Ωp dCp as a substrate in the cyclization reaction, however, we were unable to detect synthesis of $dC^{3'}pp^{5'}A$ under conditions which led to complete conversion of Ωp Cp to ΩpC>p in HeLa cell extract (Fig. 5B). This may indicate that the 2'-hydroxyl group is important for formation of putative adenylylated intermediate by the HeLa cell RNA 3' phosphate cyclase. While ATP apparently is not required for circularization of Ω>p, it has a stimulatory effect (1.5-3.0 fold), although hydrolysis seems not to be involved (unpublished results). Verification of the role of ATP in RNA 2',3'-cyclic phosphate formation and RNA ligase function requires studies on the purified activities.

Based on the results of this work we suggest that the HeLa ligase uses as substrates RNAs containing 2',3'-cyclic phosphate termini and that 3'-phosphomonoester-terminated molecules can be circularized only after prior conversion to the cyclophosphate form. The latter conclusion is supported by kinetic experiments and by the observation that ΩpdCp or $\Omega^{2'}p$, molecules which cannot undergo cyclization, are not substrates in the ligase reaction. Again, purified enzymes are required to establish this point unequivocally.

HeLa cell RNA ligase differs from the wheat germ activity (11,12). Although both enzymes use substrates with 2',3' cyclic phosphate, in the HeLa reaction this phosphate is incorporated into the 3',5'-phosphodiester linkage while in wheat germ it is recovered as a 2'-phosphomonoester in $N\langle^{p}_{p}\rangle N$ linkage (11,12). No evidence was found for $N\langle^{p}_{p}\rangle N$ formation in HeLa extracts prepared as described in Methods or according to Manley et al (20) by using as substrates Ω> $\overset{*}{p}$ and $\overset{*}{p}$Ω> p (Fig. 1) or oligo(A) bearing 5'-^{32}P-phosphate and unlabeled 2',3'-cyclic phosphate or 3'-phosphate termini (unpublished results). Failure to detect $N\langle^{p}_{p}\rangle N$ was not due to removal of the 2'-phosphate in the extract since circular Ω molecules containing 2'-^{32}P-phosphate as $N\langle^{p}_{p}\rangle N$ retained most of the label when incubated in HeLa cell extract under the same conditions (unpublished results). Ligation of the 5'-^{32}P-labeled oligonucleotides in the HeLa extract was not detected while all 5'-hydroxylated substrates, including $\overset{*}{p}$Cp-

labeled oligo(A) (data not shown), were circularized. This is in sharp contrast to the wheat germ RNA ligase which circularizes 5'-phosphorylated substrates (12).

We have recently determined that N<p>p>N is not formed during tRNA splicing in HeLa cell extract. The tRNA halves are joined instead by incorporation of the 3'-terminal phosphate of the 5' half molecule into a normal 3',5'-phosphodiester bond. Additionally, 2',3'-cyclic phosphate was observed at the 3' ends of the tRNA 5'-halves that accumulated during splicing. It could not be established whether the cyclic form is a primary product of splicing endonuclease or results secondarily from the action of RNA 3'-terminal phosphate cyclase (23). Otsuka et al. (24) have reported that partially purified splicing endonuclease from Xenopus oocyte nuclei produces tRNA 5'- and 3'-halves with 3'-phosphate and 5'-hydroxyl termini, respectively, but it was not determined whether 3' terminal phosphate was a monoester or cyclic diester. Because no processes other than RNA ligation are presently known to require terminal 2',3'-cyclic phosphate, we propose that RNA 3' phosphate cyclase may function to generate (and/or maintain) cyclic structures at the 3' ends of ligase substrates. As to the physiological role of the RNA ligase described in this work, the similarity between Ω and tRNA half molecule ligation (23) suggests that it may be involved in tRNA processing in animal cells.

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Abbreviations: Ω, RNase T1-resistant 73 nucleotide fragment of tobacco mosaic virus RNA; Ω_c, circular form of Ω; pN, nucleoside-5'-monophosphate; Np, nucleoside-3'(2')-monophosphate; $N^{2'}p$, $N^{3'}p$ and N>p, nucleoside 2'-monophosphate, 3'-monophosphate and 2',3'-cyclic phosphate, respectively; $p\overset{*}{\Omega}>p$, 5'-[^{32}P]-phosphorylated Ω enriched in 2',3'-cyclic phosphate termini; $\Omega^{3'*}p$, $\Omega^{2'*}p$ and $\Omega>\overset{*}{p}$, 5'-hydroxyl Ω with 3'-terminal [^{32}P]$G^{3'}p$, [^{32}P]$G^{2'}p$ and [^{32}P]G>p, respectively; $\Omega\overset{*}{p}Cp$ and $\Omega\overset{*}{p}dCp$, 5'-hydroxyl Ω 3'-end-labeled with [5'-^{32}P]pCp and [5'-^{32}P]pdCp, respectively. In each case the asterisk indicates ^{32}P-phosphate.

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