RNA-linked nascent DNA pieces in phage T7-infected *Escherichia coli*. III. Detection of intact primer RNA

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ABSTRACT RNA-linked DNA fragments of T7-infected *Escherichia coli* were labeled with [32 P]orthophosphate in vivo. The RNA segments of the labeled fragments were isolated by degrading the DNA portion with the 3'→5' exonuclease intrinsic to bacteriophage T4 DNA polymerase and fractionated according to net charge by a DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea. Tri-, tetra- and pentanucleotides were obtained which have ATP residues at their 5' ends. Most of the pentanucleotides had a single deoxynucleotide at the 3' end but a minor portion was totally an oligoribonucleotide. In the light of prior results, the former is a co-oligomer of an intact tetra-ribonucleotide primer and a monodeoxynucleotide and the latter is an intact penta-ribonucleotide primer. Tri- and tetra-ribonucleotides with ATP at the 5' ends had no deoxynucleotide at the 3' ends, therefore it is not clear if intact tri-ribonucleotide primers are present. The 5'-terminal dinucleotides of the tetra- and pentanucleotides were mostly pppApC and a trace amount of pppApA was present.

INTRODUCTION

A short stretch of RNA is linked to the 5' end of the nascent DNA fragment isolated from several prokaryotic systems in vivo (1-3). The RNA functions most probably as a primer which initiates discontinuous replication of DNA. RNA priming of DNA replication has been widely demonstrated in vitro in various prokaryotic as well as eukaryotic systems (4). The structure of the RNA portion of the RNA-linked DNA fragments synthesized in vivo in the prokaryotic systems has been analyzed in this laboratory by labeling the 5' termini of the RNA-linked DNA fragments with T4 polynucleotide kinase and [γ - 32 P]ATP after removal of the pre-existing phosphate groups with bacterial alkaline phosphatase (2, 5-7). The RNA-linked DNA fragments of bacteriophage T7 have been isolated and analyzed by the terminal-labeling method using T7 ts136 (gene 6)-infected *E. coli* C-N3 (polA480) system which accumulates RNA-linked DNA fragments (2, 7). The following results were obtained. The size of the RNA portion distributes from mono- to pentanucleotide. The pentanucleotide was less than all others. The 5'-terminal nucleotide of penta-, tetra- and trinucleotides are predominantly pA, pA, and pC, respectively and

pApC is the main 5'-terminal dinucleotide of tetra- and pentanucleotides. Mono- and dinucleotide are not unique, although rich in pA and pC. These results suggest that the size heterogeneity of the RNA segment may be result from in vivo degradation starting from the 5'-termini of the penta- and tetra-nucleotides.

In the present study, we have labeled in vivo the T7 RNA-linked DNA fragments with [^{32}P]orthophosphate to elect the 5'-triphosphate diagnostic of intact primers. We found pppApC (and trace amount of pppApA) at the 5' termini of tetra-ribonucleotide primers. The presence of a penta-ribonucleotide primer with the same 5' terminal nucleotide sequence is also suggested.

MATERIALS AND METHODS

Bacterium, bacteriophage and culture conditions

E. coli C-N3 (his, polA480) and bacteriophage T7 ts136 (ts mutant of gene 6) have been described (8). Cells were grown in low-phosphate M9 medium (9) supplemented with 0.5% dephosphorylated Casamino acids. The medium contained 12 μM and 57 μM of inorganic and total phosphate, respectively as assayed by the method of Martin and Doty (10). For the cultivation of bacteria, potassium phosphate buffer (pH 7.3) was added to the medium to give a final inorganic phosphate concentration of 0.5 mM. To dephosphorylate Casamino acids, 50 ml of 20% Casamino acids (Difco, certified), 8 ml of 1 M MgCl_2 and 1.8 ml of conc. NH_4OH were mixed with stirring. After 3 hr at 0°C the resulting precipitate was removed by filtration. The filtrate was stored overnight in vacuo to evaporate ammonia. The pH of the solution was adjusted to 7.3 with HCl. The volume was adjusted to 50 ml with water. To infect the bacteria with phage T7, cells grown to $6 \times 10^8/\text{ml}$ at 30°C were collected by centrifugation, suspended in an equal volume of the same medium without potassium phosphate buffer, and after shaking for 10 min, the culture was infected with T7 at a multiplicity of 10. The pattern of DNA synthesis after infection was essentially identical to that in cells grown in normal M9 medium supplemented with 0.5% Casamino acids, as judged by the incorporation rate of [^3H]thymidine and the sedimentation pattern of pulse-labeled DNA.

Enzymes and radioactive compounds

T4 DNA polymerase, and snake venom phosphodiesterase have been described (5). Penicillium citrinum nuclease P1 and E. coli RNA polymerase (holoenzyme) were kindly provided by Drs. A. Kuninaka (Yamasa Shoyu Co.) and A. Ishihama (Kyoto University), respectively. Nuclease SW was purchased from Seikagaku

Kogyo, Co. $\text{H}_3^{32}\text{PO}_4$ (carrier-free) was obtained from New England Nuclear Corp.

Labeling and isolation of short DNA fragments

E. coli C-N3 (polA480) (80 ml culture) was grown and infected with T7 ts136 as described above. At the time of infection, 25 mCi of $\text{H}_3^{32}\text{PO}_4$ was added to the culture. At 19 min after infection, the culture was transferred to 43°C and 2 min later, poured to an equal volume of an ethanol-phenol mixture. Purification of the short DNA fragments from the collected cells was carried out as described (5) except for the following. The nitro-cellulose fraction was dialysed against 5 mM Tris·HCl (pH 7.5)-1 mM EDTA, heated for 2 min at 90°C and banded in a Cs_2SO_4 density gradient. Banding was repeated two more times; before each centrifugation, the pooled DNA fractions were dialysed and heated as above. After the final centrifugation, the pooled DNA fractions (4 ml) were dialysed against 5 mM Tris·HCl (pH 7.4)-1 mM EDTA, concentrated to 1.6 ml, heated for 2 min at 90°C and passed through a column (1.54 x 29 cm) of Sephadex G-100. The excluded materials from the gel were concentrated to 0.5 ml and dialysed against 10 mM Tris·HCl (pH 8)-0.1 mM EDTA.

Enzyme reactions

Condition for the 3'→5' exonuclease of T4 DNA polymerase has been described (7). The reaction mixture (20-25 μl) for nuclease P1 contained 20 mM sodium acetate (pH 5.5), 10-100 nmol of oligonucleotides and 10 μg of the enzyme. The digestion was carried out at 37°C for 90 min. Digestion of [5'- ^{32}P]oligonucleotides with nuclease SW was carried out in a mixture (100 μl) containing 50 mM sodium carbonate buffer (pH 10.4), 0.1 M NaCl, 2 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 100 nmol of oligonucleotides and 60 units of the enzyme for 6 hr at 37°C. Complete digestion with snake venom phosphodiesterase was carried out in a mixture (24 μl) containing 30 mM glycine·KOH buffer (pH 9.0), 2.5 mM MgCl_2 , 70-140 nmol nucleotides and 10 μg of the enzyme for 90 min at 37°C.

Chromatography

DEAE-Sephadex A-25 and borate gel[†] column chromatography were carried out as described (5, 7). PEI-cellulose thin layer plates (Polygram Cel 300 PEI, Macherey-Nagel, Germany) were washed with 1.5 M potassium phosphate (pH 3.4) and then with water before use. The developing solvent for the nucleotide separation was 0.85 M potassium phosphate (pH 3.4) (11).

Other materials and methods

Autoradiography was carried out as described (7) for 2 to 5 days. Desalting was carried out as described (5). Recovery of nucleotides from PEI-cellulose thin layer plates has been described (12). Preparation of

optical density oligoribonucleotide reference has been described (7). Di-through tetranucleotides with [γ - ^{32}P]ATP at the 5' ends were prepared by digesting with nuclease SW RNA which was synthesized *in vitro* with [γ - ^{32}P]ATP as labeled substrate. Reaction mixture for the RNA synthesis (1 ml) contained 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 0.5 mM MnCl_2 , 5 mM MgCl_2 , 400 μM each of CTP and UTP, 80 μM each of GTP and [γ - ^{32}P]ATP (4×10^6 cpm/nmol), 130 μg of heat denatured T2 DNA and 200 μg of *E. coli* RNA polymerase. After the reaction at 37°C for 4 hr, nucleic acids were purified by phenol treatment and gel filtration through Sephadex G-50. Digestion with nuclease SW was carried out in a reaction mixture (750 μl) as described above with 60 units of the enzyme for 4 hr at 37°C.

RESULTS

Alkaline hydrolysis of ^{32}P -labeled short DNA fragments

^{32}P -labeled short DNA fragments were first subjected to alkaline hydrolysis. About 1% of the radioactivity became acid soluble by the treatment. The hydrolysate was fractionated by a column chromatography of DEAE-Sephadex A-25 in the presence of 7 M urea (Fig. 1). Radioactivity eluted at the mononucle-

Fig. 1. DEAE-Sephadex A-25 column chromatography of [^{32}P]short DNA fragments after alkaline hydrolysis. [^{32}P]short DNA fragments were prepared as described in Materials and Methods except that the 2nd gel filtration on Sephadex G-100 was omitted. They were subjected to an alkaline hydrolysis (0.3 N KOH, 37°C x 18 hr). After neutralization with Bio-Rad AG50W (H^+) resin, the hydrolysate was fractionated by a column (0.24 x 22 cm) of DEAE-Sephadex A-25 in the presence of 7 M urea. Elution was carried out with a 120-ml linear gradient of NaCl (0-0.3 M) in 10 mM Tris-HCl (pH 7.5)-7 M urea. 0.1 μmol of 5'-AMP was added as O.D. marker. —●—●—, ^{32}P radioactivity; —○—○—, absorbance at 260 nm.

otide position (fractions 13 to 29, containing 0.8% of the input radioactivity) was analysed by a thin layer chromatography on a PEI-cellulose plate and the composition was 34.5% in 2'(3')-GMP, 29.0% in 2'(3')-AMP, 18.7% in 2'(3')-CMP and 17.8% in 2'(3')-UMP. The peak of radioactivity in fractions 49 to 62 (containing 0.2% of the input radioactivity), corresponding to the elution position of pNp, was also analysed by a PEI-cellulose thin layer chromatography and the composition was 56.0% in pCp, 21.4% in pAp, 12.5% in pGp and 10.1% in pUp. The last peak (fractions 70 to 80 containing 0.2% of the input radioactivity) corresponds to the position of pppNp. It was further treated with nuclease P1 which cleaves the terminal 3'-phosphate but not the terminal 2'-phosphate produced by alkaline hydrolysis of RNA (13). The products were undegraded material (38 cpm), ATP (60 cpm) and Pi (17 cpm) as analysed by a PEI-cellulose thin layer chromatography. This demonstrates molecules with ATP at 5'-termini. The ratio of triphosphoryl terminated molecules to (ribo)monophosphoryl terminated ones was roughly 1:2.

Digestion of ^{32}P -labeled short DNA fragments with the 3' $\rightarrow$ 5' exonuclease associated with T4 DNA polymerase

To examine the chain length of the RNA segment which has a 5'-triphosphate terminus, ^{32}P -labeled short DNA fragments were digested with the 3' $\rightarrow$ 5' exonuclease associated with T4 DNA polymerase. RNA-linked DNA fragments are degraded to RNA-deoxymononucleotides and a minor portion of free RNA by this enzyme (2, 6, 7). After digestion of the labeled nascent fragments by this enzyme, the digest was fractionated according to the net charge by a column of DEAE-Sephadex A-25 in the presence of 7 M urea (Fig. 2). Ninety-nine percent of the recovered radioactivity was eluted at the mononucleotide region, followed by the elution of 6 discrete peaks. The number of peaks is different from that observed when the T7 RNA-linked DNA fragments were labeled with ^{32}P at the 5' end with polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, where peaks up to only hexanucleotide were observed and only a trace of hexanucleotide was found (7). This could be the result of 5'-tri(or di)phosphoryl terminated molecules in the larger oligonucleotide fractions of the digest of *in vivo* labeled fragments. The positions of peaks 4, 5, 6 and 7 correspond to those of di-, tri-, tetra- and pentanucleotide with 5'-triphosphate end, respectively. Peaks 3 through 7 were then subjected to the chromatography on a borate gel column as described in the preceding paper (7). As shown in Fig. 3, most of the radioactivity in peaks 3, 4 and 7 did not attach to the gel, indicating that these molecules have deoxynucleotides at the 3' ends. On the other hand, half of the radioactivity in peaks 5 and 6 were retained to the gel, indicating that

Fig. 2. DEAE-Sephadex A-25 column chromatography of [^{32}P]short DNA fragments after digestion with the T4 3' $\rightarrow$ 5' exonuclease. Short DNA fragments labeled with $\text{H}_3^{32}\text{PO}_4$ *in vivo* were purified and digested with the exonuclease of T4 DNA polymerase. Ninety percent of the radioactivity was rendered acid soluble by the reaction. The digests in 10 mM Tris-HCl (pH 7.5)-7 M urea were applied to a column (0.16 x 90 cm) of DEAE-Sephadex A-25. Elution was carried out with a linear gradient (230 ml) of 0.03-0.32 M NaCl in 10 mM Tris-HCl (pH 7.5)-7 M urea. Partial digest of *E. coli* tRNA with nuclease SW was co-chromatographed as size markers. The positions of di- to tetranucleotides with ATP at the 5' ends were determined in a separate experiment. —●—●—, ^{32}P radioactivity; - - - - - , absorbance at 260 nm.

these molecules have RNA termini, presumably produced by digestion up to or beyond the RNA-DNA junction by the 3' $\rightarrow$ 5' exonuclease associated with T4 DNA polymerase.

Detection of 5'-triphosphate termini

Subfractions of peaks 3 to 7 which were not retained or retained to the borate gel (referred to a and b, respectively) were then examined for the presence of a 5'-triphosphate end by PEI-cellulose thin layer chromatography after digestion with nuclease P1. This enzyme degrades DNA and RNA to 5'-mononucleotides (13) and 5'-terminal triphosphates are recovered as nucleoside 5'-triphosphates. Spots of radioactivity appeared at the position of ATP in the samples 7a, 7b, 6b and 5b (Fig. 4). The spots are in fact ATP, since snake venom phosphodiesterase split them into AMP and pyrophosphate (Fig. 5). Taking account of the specificity of the 3' to 5' exonuclease of T4 DNA

Fig. 3. Borate gel column chromatography of oligonucleotides. Fractions 3 to 7 in Fig. 2 were pooled separately, desalted and subjected to the borate gel column chromatography as described in Materials and Methods. —●—●—, ^{32}P radioactivity; —○—○—, absorbance at 260 nm.

polymerase, 5'-triphosphoryl terminated molecules recovered in fractions 7a, 7b, 6b and 5b are co-oligomers of tetra- and monodeoxynucleotide, pentaribonucleotide, tetra- and triribonucleotide, respectively (see Discussion). Appearance of ATP from fraction 7a indicates that intact primer exists whose structure is ATP-terminated tetra-ribonucleotides.

Identification of 5' terminal di- and tri-nucleotide

Nuclease SW degrades polynucleotides longer than tetranucleotide to 5'-phosphoryl terminated di- and trinucleotides (14). A triphosphate group at the 5'-end of RNA is not attacked by the enzyme (T. Ogawa, unpublished observation). Fractions 7a and 6b were digested by this enzyme and the products were displayed by DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea. Fractions eluting with 5'-triphosphoryl terminated dinucleotides, and trinucleotides from the 7a digests, were degraded to the constituent mononucleotide with nuclease P1 and chromatographed on a PEI-cellulose plate (Fig. 6). Beside the ATP spot, there were a pC and a trace of pA spots. Thus, pppApC and a trace of pppApA are the 5'-terminal dinucleotide sequences of the primer RNA in fractions 7a and 6b. The third nucleotide in from the 5' end of the primer RNA in fraction 7a is also mostly CMP.

Fig. 4. Autoradiogram of a PEI-cellulose thin layer chromatogram of oligonucleotides after digestion with nuclease P1. Borate gel-retained (b) or not retained (a) subfractions of fractions 3 to 7 in Fig. 3 were pooled separately, desalted and treated with nuclease P1. The digests were subjected to a PEI-cellulose thin layer chromatography along with unlabeled mixture of the 4 common rNMP's and ATP.

Fig. 5. PEI-cellulose chromatogram of venom phosphodiesterase digest of terminal ATP moiety of fragments. Radioactivities at the positions corresponding to ATP in the chromatogram of Fig. 4 were recovered from the plate, treated with snake venom phosphodiesterase and subjected to a PEI-cellulose thin layer chromatography as described in Fig. 4. 1, $\text{Na}_4^{32}\text{P}_2\text{O}_7 + \text{H}_3^{32}\text{PO}_4$; 2, Fraction 5b; 3, Fraction 6b; 4, Fraction 7 (a + b).

Fig. 6. Autoradiogram of a PEI-cellulose thin layer chromatogram of nuclease P1 digest of di- and trinucleotides with ATP at the 5' end. Portions of fractions 7a and 6b were treated with nuclease SW and fractionated by a column of DEAE-Sephadex A-25 in the presence of 7 M urea. Peaks of radioactivity of pppApN and, in the case of fraction 7a, pppApNpN were pooled, desalted, treated with nuclease P1 and subjected to a PEI-cellulose thin layer chromatography as described in Fig. 4. 1, dinucleotide from fraction 7a; 2, trinucleotide from fraction 7a; 3, dinucleotide from fraction 6b.

DISCUSSION

Two methods have been employed previously in this laboratory for the detection of the RNA-linked nascent DNA fragments *in vivo*. The spleen exonuclease method (15) is specific to nascent, pulse-labeled DNA, but it is indirect, since 5'-hydroxyl terminated DNA molecules are measured after removal of the RNA portion by the hydrolysis with alkali or RNases. The polynucleotide kinase method (16, 5) is very useful for the structural analyses of the RNA-linked DNA fragments since the 5' end can be very highly labeled. However, since the method is not specific to the nascent molecules, contaminating irrelevant RNA-linked DNA molecules would confuse the analyses. Furthermore, intact primer RNA containing a 5'-triphosphate group cannot be detected by the method, since all the pre-existing terminal phosphates are removed by the bacterial alkaline phosphatase before labeling. To circumvent these problems, in the present study, we isolated RNA-linked nascent DNA fragments labeled with $H_3^{32}PO_4$ *in vivo* using the T7-infected *E. coli* system. RNA molecule which has 5'-triphosphate and is covalently linked to the 5' end of DNA, if present, would be an intact primer for DNA synthesis. Presence of intact primer labeled with $H_3^{32}PO_4$ after infection, eliminate the possibility that they are derived from pre-existing oligoribonucleotides.

Since the amount of radioactivity incorporated into the primer RNA is

very small, it is essential to eliminate the huge radioactive noise derived from irrelevant molecules as completely as possible. For the purification of nascent fragments, two successive centrifugations in Cs_2SO_4 and gel filtration on Sephadex G-100 were carried out after the purification procedure which was employed to prepare the nascent fragments for the analyses using polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5). The use of the 3' $\rightarrow$ 5' exonuclease of the T4 DNA polymerase for the isolation of the RNA segments also helps to obtain pure RNA molecules since most of the free DNA are degraded to mononucleotides and 5'-terminal dinucleotides by this enzyme. Contamination of RNA molecules to the oligonucleotides analysed in the present study (fractions 3 to 7 in Fig. 2) is satisfactorily low. Most of them had been removed during the extensive purification procedure. A trace of free RNA molecule, if present in the preparation of RNA-linked DNA fragments after purification, would not confuse the analyses, since most of the RNA molecules which survive during extensive purification procedures by interacting non-covalently with DNA would be longer than the oligonucleotides analysed here and they should not be degraded by the 3' $\rightarrow$ 5' exonuclease activity of T4 DNA polymerase (5, 6). Therefore, not only the oligoribonucleotides tagged with a single deoxynucleotide at the 3' end but also most of the free oligoribonucleotides in the T4 DNA polymerase digest seem to be derived from the RNA-linked DNA molecules. AC rich composition of the molecules supports this idea (Fig. 4). Nucleoside monophosphates produced by an alkaline hydrolysis of the less purified preparation of ^{32}P -labeled short DNA fragments (Fig. 1) were rich in GMP rather than AMP and CMP. They might have originated from contaminating RNA.

ATP-terminated molecules were detected in four fractions of primer RNA; fraction 7a, 7b, 6b and 5b (Fig. 4). ATP-terminated molecules in fraction 7a should have a deoxynucleotide at the 3' end since they were not retained by the borate gel column. They have a net charge of -8 judging from the elution position in Fig. 2. Therefore, they are co-oligomers consisting of ATP-terminated tetra- and monodeoxynucleotide, corresponding to the tetra- and monodeoxynucleotides in the pentanucleotide fraction, having a 5'-terminal dinucleotide sequence of pApC in the previous study (7). Thus, the presence of intact tetra- and monodeoxynucleotide primer has been established. ATP-terminated molecules in fraction 7b are pentaribonucleotides since they have a net charge of -8 and have no deoxynucleotide at their 3' end. These molecules are probably intact primers corresponding to the pentaribonucleotide with a single deoxynucleotide at the 3' end which was analysed previously (7),

since the latter was the largest primer in the T4 DNA polymerase digest and had an AMP residue at the 5' end. Therefore, the presence of intact pentaribonucleotide primer also seems to be confirmed. The amount of these molecules is several times smaller than the ATP-terminated tetraribonucleotide primer. ATP-terminated tri- and tetraribonucleotides in fraction 5 and 6, respectively had no deoxynucleotide at their 3' termini. These molecules also seem to be derived from the RNA-linked DNA fragments for the reasons discussed above. This was surprising as the bulk of the digestion product of RNA-linked DNA fragment by the 3'→5' exonuclease associated with T4 DNA polymerase are generally an RNA-deoxymononucleotide. The proportion of the borate gel-adsorbable molecules did not decrease when the digestion was carried out in a more mild condition under which 75% of the radioactivity was rendered acid soluble (data not shown). The reason for this is unknown, thus it is not clear whether the ATP-terminated tri- and tetraribonucleotides are intact primers or processed from larger molecules.

Some spots of radioactivity were observed which did not coincide with that of the optical density references in the PEI-cellulose chromatogram of the nuclease P1 digest (Fig. 4). Further characterization has not been performed on these spots. The positions of the spots near the origin in the chromatogram of P1 digests of fractions 3 and 4 do not coincide with that of any of the 4 common rNTP's. They might be undegraded oligonucleotides or compounds other than usual nucleotides.

A limitation intrinsic to the $H_3^{32}PO_4$ -labeling method in vivo is the difficulty in obtaining enough radioactive label in the primer RNA portion. The specific radioactivity of ^{32}P was one hundredth of that which can be obtained in the experiments using polynucleotide kinase and $[\gamma-^{32}P]ATP$. Therefore, extensive analyses of the structure of the primer RNA molecules could not be performed. Moreover, the accurate calculation of nucleotide composition in primer RNA is impossible since the specific radioactivity of each nucleotide may vary. Nucleoside monophosphate produced by the nuclease P1 digestion of the borate gel-adsorbable oligonucleotides are rich in AMP and CMP, poor in UMP and deficient in GMP (Fig. 4). Mononucleotides produced from the borate gel-non-adsorbable oligonucleotides showed similar nucleotide composition but increased in the radioactivity at the position of UMP. Contribution of deoxynucleotide residues in the digest of the borate gel-non-adsorbable oligonucleotides is also unknown. However, the results are in keeping with the AC rich composition of primer RNA obtained with 5'-terminally labeled preparations.

Recently, Scherzinger et al. (17) and Richardson et al. (18) have purified the product of T7 gene 4 which is essential for the phage DNA replication in vivo and in vitro. In the presence of ATP, CTP, DNA-binding protein and single-stranded template DNA, the gene 4 protein synthesized tetranucleotide primers having the sequence of pppApCpCpA (17) or pppApCpCpC (18), which are covalently extended with deoxynucleotides by T7 DNA polymerase. The structure of the primer made in vitro and in vivo are very similar but the chain length and the nucleotide sequence of the primer made in vivo are more diverse.

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† Abbreviations used have been described in the preceding paper (7).

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