Vol. 82, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 14, 1978 Pages 1040-1048

# SELECTIVITY OF TRANSCRIPTION AND STRUCTURE OF COLIPHAGE N4 VIRION-ASSOCIATED RNA POLYMERASE

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Received September 19,1977

SUMMARY. - The protomer of coliphage N4 virion-associated RNA polymerase,isolated through a gentle purification procedure and analyzed by zone sedimentation in glycerol gradients and SDS polyacrylamide gel electrophoresis, is composed of four apparently identical subunits and has a molecular weight of 200,000 daltons. This form of the enzyme is extremely specific and transcribes efficiently only native N4 DNA and the homopolimer dG . dC yielding poly rG. Under most *in vitro* conditions however, the protomer is very labile and dissociates into stable subunits of molecular weight 50,000. The monomeric enzyme, while retaining the property of synthesizing RNA, shows a complete lack of selectivity of transcription.

Coliphage N4, a morphologically complex phage (1) containing double stranded DNA of molecular weight $40 \times 10^6$ daltons (2) whose genetics and physiology have been studied in some detail (3-9), provides the first example of a bacterial virus endowed with a virion-associated transcription apparatus. We have shown recently, in fact, that purified N4 particles contain a rifampicin-resistant DNA-dependent RNA polymerase ( vRNAP ) which, when injected into the host cell, initiates direct transcription of the phage genome (10). Data presented previously also indicated that N4 vRNAP actively copies *in vitro* only the homologous DNA template (10). We report here that the native form of N4 vRNAP is a polymeric enzyme which easily dissociates into subunits. These monomers, while retaining the property of synthesizing polyribonucleotide chains, show a complete loss of selectivity of transcription.

## MATERIALS AND METHODS

Bacteriophage N4 was grown and purified as previously described (4). Purified suspensions, containing about 20-25 mg virus per ml, were disrupted by three cycles of freezing and thawing in liquid nitrogen and the viscous

0006-291X/78/0823-1040$01.00/0

cryolysates were sonicated for 30 seconds in a Branson apparatus at an output of 50 W. The standard reaction mixture for assay of N4 vRNAP contained 10 μmol Tris-HCl (pH 7.9), 2.5 μmol $MgCl_2$, 25 μmol dithiothreitol, 25 nmol EDTA, 125 μg bovine serum albumin, 2 pmol native N4 DNA, 37.5 μmol each of the four ribonucleotide triphosphates of which UTP was labelled with $^3H$ ( specific activity $5x10^3$ - $2x10^4$ c.p.m. per nmol ) and 30 μl enzyme as specified further in a final volume of 250 μl. After incubation at 37° for 15 min the acid-insoluble radioactivity was collected and counted as reported previously (7). Phage DNAs were prepared by phenol extraction (2) and other DNAs employing the procedure developed by Marmur (11). Single stranded DNAs were obtained by thermal denaturation (12). Sedimentation analysis of N4 vRNAP was performed in linear glycerol gradients, under the conditions detailed in the text, in the SW-41 rotor of a Spinco L3-50 ultracentrifuge. SDS-polyacrylamide electrophoresis in 10% slab gels was carried out using the procedures and apparatus described previously (13).

## RESULTS AND DISCUSSION

### Molecular weight and specificity of native N4 RNA polymerase.

Preliminary indications on the molecular structure of N4 vRNAP suggested that the enzyme, under denaturating conditions, was associated with a polypeptide chain of 50 to 60,000 daltons (10). Attempts to characterize the enzyme under more physiological conditions were hampered by the fact that following disruption of the phage the activity remained associated with viral DNA and that all synthetic ability of the cryolysates was lost in less then 24 hours. The finding that glycerol at a concentration of 10-20% stabilized N4 vRNAP for at least 3 days rendered possible further study of the enzyme. Dissociation of the native vRNAP from its template was achieved by increasing the ionic strength (14) of the cryolysate followed by zone sedimentation in glycerol gradients and dialysis to restore critical storing conditions. In a typical experiment, 1 ml of N4 cryolysate was adjusted to contain 0.04 M tris-HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 M KCl and 10% glycerol ( Buffer A) and kept at 4° for 4 h. After clarification at 20,000 rpm for 40 minutes 0.7 ml aliquots of the supernatant fluid were layered on a 15-30% glycerol gradient made up in Buffer A and spinned at 32,000 rpm for 16 h. Individual 0.5 ml fractions were then dyalized at 4° for 4 h against a solution identical to Buffer A except for the absence of KCl and tested for RNA-polymerizing activity with native N4 DNA as primer. Under these conditions the enzyme was totally dependent on added DNA and, as shown in Fig. 1a, migrated as a single peak. Calibration of the gradient

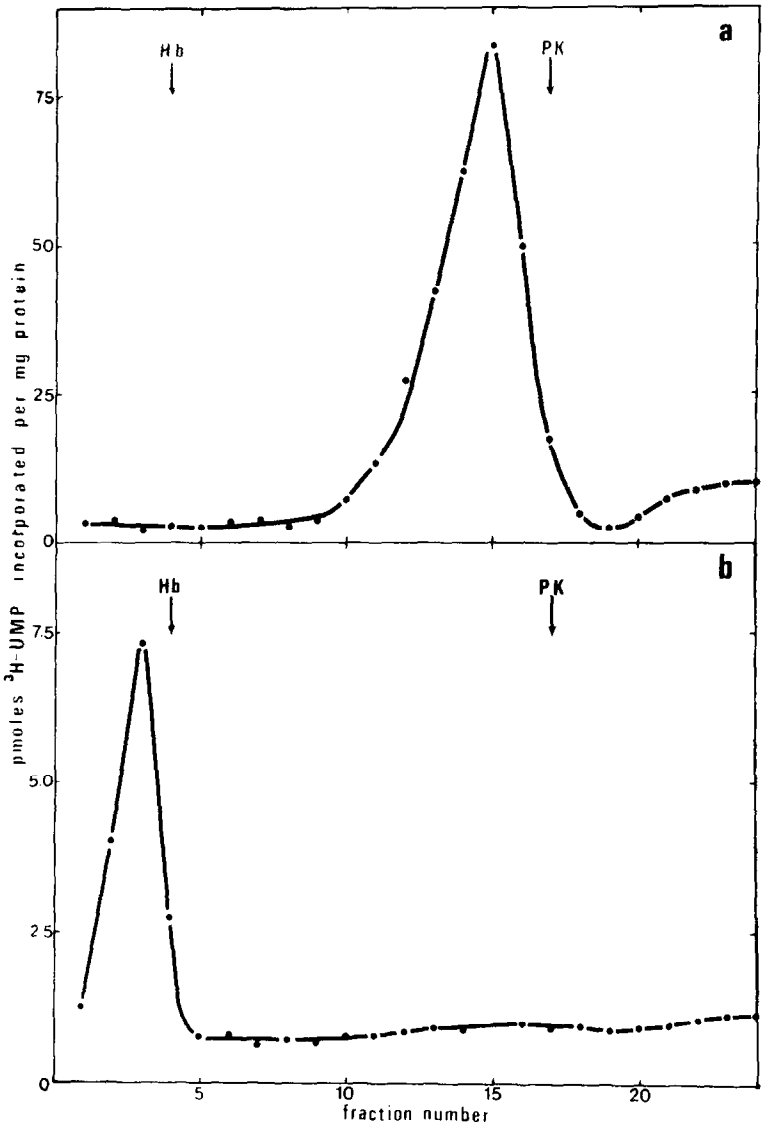


Fig. 1. Zone sedimentation of N4 virion associated RNA polymerase. Conditions of centrifugation are specified in the text. Sedimentation standards human hemoglobin (Hb, $s_{20,w}$=4.13 S) and piruvate kinase (PK, $s_{20,w}$-10.0 S), were run in parallel in a duplicate gradient. Fractions of 0.50 ml were collected and assayed for RNA polymerase activity with N4 DNA as primer;a: high molecular weight enzyme in Buffer A containing 0.04 M Tris buffer, pH 7.9; b: low molecular weight enzyme in Buffer A containing 0.01 M Tris buffer, pH 7.9.

(15) using human hemoglobin (16) and pyruvate kinase (17) as sedimentation standards gave an estimate of $S_{20,w}$ of 8.75 S for N4 vRNAP. Assuming a globular configuration and a $\bar{v}$ of 0.73 ml per g (18) this figure corresponded

to a molecular weight of about 210,000 daltons.

The reaction requirements of the purified enzyme were identical to those already reported for the crude activity present in N4 cryolysates (10). N4 vRNAP confirmed its previously reported remarkable template specificity (Table 1) since it copied only N4 DNA. Denatured N4 DNA, calf thymus, *E.coli*, T4, and λ DNAs were almost inactive as primers. The dAT copolymer was also an unsuitable template for N4 enzyme. On the other hand the homopolymer pair dG . dC was moderately active for poly rG synthesis but could not direct poly rC production suggesting, as for T7, T3 and gh-1 enzymes (19-21), that N4 vRNAP is highly specific in its recognition of initiation sites on DNA.

Subunits of N4 RNA polymerase lack specificity of transcription.

Glycerol gradient analysis indicated that N4 vRNAP is a large protein with a molecular weight of about 210,000. This result taken together with previous data of SDS-gel separation (10) suggested that N4 enzyme might be built up by several polypeptides. Attempts were therefore aimed at the isolation of such subunits under mild conditions. It was soon realized that the native form of N4 vRNAP, as it probably occurs in the virion, was an extremely labile molecule with a strong tendency to dissociate into catalitically active more stable ( at least one month in 25% glycerol at -20°) but rather aspecific subunits. When the enzyme was purified from cryolysates through zone sedimentation in a glycerol gradient made up in a solution identical to Buffer A except for the concentration of Tris buffer (pH 7.9) which was lowered to 0.01 M, analysis of the fractions revealed a single peak of RNA polymerizing activity (Fig. 1 b) sedimenting with an $S_{20,w}$ value of 3.36 S corresponding (18) to a molecular weight of about 50,000 daltons. No trace of the 8.75 S component was detected under these conditions. Similar results were obtained by shifting the concentration of Tris in Buffer A to 0.08 M. Minor variations in the pH of Buffer A also gave rise to total breakdown of the high molecular weight form of the enzyme. The range of existence of N4 vRNAP seems therefore extremely restricted and this rather unexpected property may explain the failure of other workers to detect the native enzyme in 4 M guanidine extracts of N4 particles(22).

Since the subunits obtained under mild conditions were still able to produce polyribonucleotide chains this activity was further characterized. Syn-

Table 1. Template specificity of N4 virion-associated RNA-polymerase

| Template DNA added | Activity | |
|---|---|---|
| | Native enzyme | Subunit enzyme |
| None | 1 | 2 |
| N4 DNA (50 µg per ml) | 100 | 100 |
| Denatured N4 DNA(50 µg per ml) | 2 | 60 |
| Calf thymus DNA (50 µg per ml) | 3 | 330 |
| *E.coli* DNA (200 µg per ml) | 1 | 81 |
| T4 DNA (50 µg per ml) | 2 | 30 |
| λ DNA (25 µg per ml) | 2 | 35 |
| dAT copolymer(20 µg per ml) | 3 | 17 |
| dG.dC homopolymer (20µg per ml) | | |
| $[^3H]$GTP | 45 | 40 |
| $[^3H]$CTP | 2 | 315 |

Conditions of assay as described in *Materials and Methods* except that, where indicated, N4 DNA was replaced with other DNAs. For the native enzyme 100% activity was 85 pmol UMP incorporated per µg protein.For the subunit enzyme 100% activity corresponded to 10 pmoles incorporated per µg protein.

thesis of RNase-sensitive acid-precipitable material was totally dependent on the presence of a primer DNA and showed the same general requirements manifested by the native enzyme (10). On the other hand, striking differences were noted when the selectivity of transcription on various DNA templates was analyzed. While native N4 vRNAP was active only with N4 DNA and dG . dC yielding poly rG exclusively, the low molecular weight enzyme copied all the native DNAs tested , denatured N4 DNA and was prominently active with calf thymus DNA and with dG . dC homopolymer yielding poly rC preferentially (Table 1).

To test the specificity of N4 vRNAP further, we determined the efficiency of hybridization to N4 denatured DNA of the RNA synthesized *in vitro* (10) by the two molecular forms of the enzyme with N4 DNA as template. Only the native form gave rise to a product annealing with high efficiency (over 50%) to N4 DNA whereas the monomers synthesized RNA species that showed a very

low order of affinity (5-8%) for their primer and a notable degree of mismatching as revealed by a large influence of RNase treatment on the efficiency of hybridization (Table 2).

Proposed quaternary structure of N4 RNA polymerase

In order to get some insights into the quaternary structure of N4 vRNAP all the fractions obtained after zone sedimentation of [$^{35}$S] - labelled (6) N4 cryolysates under conditions identical to those specified in Fig. 1a were assayed for N4 RNA-polymerase activity and analyzed by SDS polyacrylamide slab gel electrophoresis followed by autoradiography. Only the fractions with enzyme activity contained a single sharp band of molecular weight 50,000 (Fig.2). These results, taken together with the data of zone sedimentation in glycerol gradients, offer not conclusive but strong evidence that native N4 RNA polymerase with a molecular weight of about 210,000 is a homotetrameric protein containing four subunits of molecular weight about 50,000. We are unable to provide additional evidence on this point from $NH_2$-terminal amino acid or peptide analysis because of the small quantities of purified proteins available to us at present.

N4 vRNAP is a virus-coded protein since it has been possible to isolate temperature sensitive mutants of the phage which are unable to replicate at 40° *in vivo* and that show *in vitro* a thermolabile enzymatic activity (22,23). With a size of only 50,000 daltons the polypeptide specified by N4 seems to be the most primitive molecule able to inititiate and elongate RNA chains thus far studied (24) and may represent the simplest model that could yield an understanding of the chemistry of transcription. Possibly as a consequence of its simple structure N4 monomeric enzyme lacks the precise primer recognition ability shown by other virus-coded RNA polymerases (T3,T7,ghl) which are composed of a single but larger polypeptide (19-21), and initiates RNA synthesis at aspecific sites on a variety of DNA templates. Selectivity of transcription leading to the ability to read exclusively the promoter and terminator signals found on the homologous phage DNA is achieved through aggregation of the subunit enzyme to give a protomeric structure which we deem to be the functional form of N4 vRNAP. Employing this novel strategy, that spares genetic information, N4 enzyme gains an articulation in structure similar to that shown by the more evolved viral and procaryotic RNA polymerases (25).

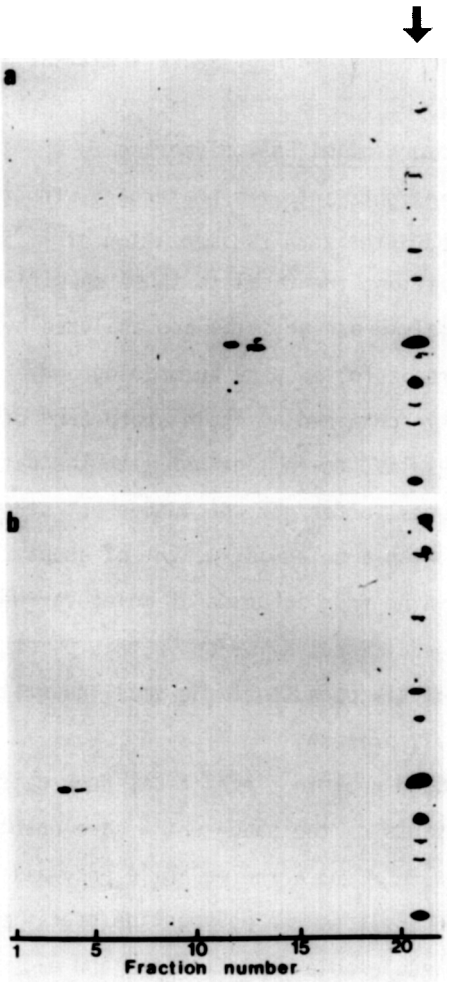


Fig. 2. Analysis of N4 RNA polymerase fractions in SDS-polyacrylamide gels. $^{35}S$ - labelled N4 cryolysates (10) were layered on to a 15-30% glycerol gradient under the conditions specified in Fig.1 (a and b) and spinned at 32.000 rpm for 16 h.
Fractions (0.6 ml) were collected and analysed for RNA-polymerase activity and [$^{35}S$] radioactivity. Samples of 25 µl of each fraction were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (13).
a) migration pattern of fractions obtained after glycerol gradient centrifugation in Buffer A containing 0.04 M Tris pH 7.9 ( native enzyme)
b) migration pattern of fractions obtained after glycerol gradient centrifugation in Buffer A containing 0.01 M Tris pH 7.9 ( subunits enzyme).
Fractions 11 and 12 (a)contained 1860 and 1100 cpm in 25 µl; fractions 3 and 4 (b) 1370 and 560 respectively. All other fractions contained less than 250 cpm in 25 µl. N4 virion polypeptides (6) (25 µl, 50000 cpm) were also run (arrow) as molecular weight standard.
N4 RNA polymerase activity was found in fractions 11 - 12 and 3 - 4 of gradient a and b respectively.

Table 2. Hybridization of N4 RNA polymerase products to N4 DNA

| Enzyme | Amount of *in vitro* RNA (c.p.m.) | c.p.m. in hybrid + RNase | - RNase |
|---|---|---|---|
| Native | 3150 | 1600 (51%) | 1710 (54%) |
| Subunits | 1450 | 87 (6%) | 406 (28%) |

Labelled RNA synthesized by N4 native or monomeric RNA polymerase was purified from the reaction mixture (10) by phenol extraction (8).
RNA-DNA hybridization was carried out as already reported (8) using N4 DNA (2μg) bound to nitrocellulose filters. RNase treatment was performed at room temperature for 1 hour at a concentration of 25 μg per ml.
These results represent the mean values of three in dependent experiments.

This complexity probably correlates with the multiplicity of control mechanisms modulating the *in vivo* expression of N4 genome. A more detailed study on the effects of the ionic environment and of the presence of the DNA template on the structure and catalytic activity of N4 vRNAP is in progress and will be reported elsewhere.

ACNOWLEDGEMENTS: This research was supported by Grant n° 75.00707.04 from the Italian Consiglio Nazionale delle Ricerche.

REFERENCES

1. Schito G.C., Pesce A. and Rialdi G. (1966) Biochim. Biophys. Acta, 129, 482-490.
2. Schito G.C., Pesce A. and Rialdi G. (1966) Biochim. Biophys. Acta, 129 , 491-501.
3. Schito G.C.(1967) Virology, 32, 723-725.
4. Schito G.C.(1973) Virology, 55, 254-265.
5. Schito G.C.(1974) J. Virol., 13, 186-196.
6. Giraldi M., Toni M. and Schito G.C.(1973) Virology, 55, 476-485.
7. Schito G.C., Giraldi M. and Toni M. (1974) Nature, 251, 340-342.
8. Rothman-Denes L.B. and Schito G.C. (1974) Virology, 60, 65-72.
9. Vander Laan K., Falco S.C. and Rothman-Denes L.B.(1977) Virology, 76, 596-601.

10. Pesce A., Casoli C. and Schito G.C.(1976)Nature, 262, 412-414.
11. Marmur J.(1961) J. Mol. Biol., 3, 208-218.
12. Schildkraut C.L., Marmur J. and Doty P.(1962) J.Mol.Biol.,4, 430-442.
13. Toni M., Conti G. and Schito G.C.(1976) Biochem. Biophys. Res. Commun., 68, 545-550.
14. Burgess R.R.(1976) in Losick R. and Chamberlin M.S. RNA polymerase pp 69-100, Cold Spring Harbor Laboratory.
15. Martin R.G. and Ames B.N.(1961) J.Biol.Chem., 236, 1372-1379.
16. Chiancone E.,Vecchini B., Forlani L., Antonini E., and Wyman J.(1966) Biochim. Biophys. Acta, 127, 549-562.
17. Cottam G.L., Hollenberg P.F. and Coon M.J.(1969) J.Biol.Chem., 244, 1481-1486.
18. Schachman H.K.(1959) Ultracentrifugation in Biochemistry, Academic Press, New York.
19. Chamberlin M.J., McGrath J. and Waskell L. (1970) Nature, 228, 227-231.
20. Dunn J.J., Bautz F.A. and E.K.F.(1971) Nature New Biol., 230, 94-98.
21. Towle H.C., Jolly J.F. and Boezi J.A.(1975) J. Biol. Chem., 250, 1723-1728.
22. Falco S.C., Vander Laan K. and Rothman-Denes L.B.(1977) Proc. Natl. Acad. Sci.USA, 74, 520-523.
23. Pesce A.(1977) FEMS Microbiology Letters (in the press)
24. Zillig W., Palm P. and Heil A.(1976) in Losick R., and Chamberlin M.J. RNA Polymerase, pp 101-125, Cold Spring Harbor Laboratory.
25. Bautz E.K.F.(1976) in Losick R., and Chamberlin M.J. RNA Polymerase, pp.273-284, Cold Spring Harbor Laboratory.