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ASSESMENT OF THE EFFECTS OF ACCESSORY REPLICATION PROTEINS ON THE 3′ to 5′ EXONUCLEASE ACTIVITY OF BACTERIOPHAGE T_4 DNA-POLYMERASE'

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The gene 32 protein of bacteriophage T4 induces a small increase in the turnover of precursor deoxynucleotide triphosphates during replication of primed single stranded X174 *am* 86 template How much of this enhanced turnover involves misincorporated bases is not determinable with a natural template containing all the four bases. Gene 45 and gene 41 proteins did not show any effect on turnove γ.

INTRODUCTION

Proteins encoded by bacteriophage T_4 genes 32, 41, 44, 45, 61 and 62 are required for accurate and efficient replication by T_4 DNA polymerase [7, 1, 18, 43, 42, 27]. The purified gene products can be reconstituted *in vitro* to form a replication complex, which under standard conditions of synthesis replicates a variety of templates with great accuracy [26, 39]. The *in vitro* replication accuracy closely parallels that seen *in vivo* [20,37].

DNA replication is a central operation in the life of an organism; hence it is accomplished with great accuracy. Molecular mechanisms controlling the fidelity of DNA replication operate through error-prevention and error-correction. Error-prevention essentially refers to the selection of the correct base while the template is being read. Correct base selection is believed to be controlled by many factors. These include hydrogen-bonding and base-stacking energies, specificity of the polymerase acting alone or in association with other proteins, composition of the nucleotide pool, the nucleotide sequence (nearest neighbours as well as distant bases in the sequence) and the DNA secondary structure [38]. Error correction refers to the removal of the mismatched base. In prokaryotes there are at least two mechanisms for correction: (i) removal of the mismatch by the proof reading function of the 3′-to-5′ exonuclease [5, 28, 14, 15, 32, 2, 11, 12, 21, 22, 3, 6]; and (ii) the post-replication mismatch correction which relies upon the methylation of the template strand [29, 44, 13].

In the absence of any contribution by the replication proteins, the discrimination between the correct and incorrect nucleotides is a function of the free energy difference (ΔG) between complementary and noncomplementary base pairs, which is generally accepted as being about 1 to 3 kilocal./mole [9, 16, 35, 8]. This would predict that there would be an error frequency of 1 mispaired nucleotide out of every 10 to 100 nucleotides incorporated [33, 30]. The observed error rate is much less than this even without a functional proof-reading mechanism [23], implying that the DNA polymerase itself is capable of discrimination.

With the proof-reading mechanism, i.e., the 3′-to-5′ exonuclease portion of the DNA polymerase, the error correction is enhanced very markedly. The proof-reading exonuclease recognizes a mismatch and edits it out, albeit at a cost. Because the 3′-to-5′ exonuclease is responding to a frayed end, it occasionally removes the correct nucleotide. The accuracy of replication is further enhanced by the accessory proteins. The most important contribution is made by the gene 32 protein. A role for this protein in modulating fidelity is suggested by studies demonstrating that mutations in the gene encoding it are accompanied by an increase in mutation rates throughout the genome (cf. Drake, 1973). The first *in vitro* demonstration came from the studies of Gillin and Nossal [11, 12] who showed that gene 32 protein reduces the turnover of non-complementary nucleotide triphosphates. Using homopolymer templates, Topal, *et al.* [40] have shown that genes 32 and 45 encoded proteins inhibit the incorporation of incorrect precursors at the end of the growing strand. They showed that the turnover of non-complementary dNTPs during the copying of poly d(AT) *in vitro* by phage

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T_4 DNA polymerase is reduced by 30-80% when the gene 32 product is present. Moreover, "partial reactions" involving different combinations of the T_4 DNA polymerase with accessory proteins demonstrate that the polymerase achieves a maximum level of accuracy when acting in concert with gp32, gp44/62, and gp45 [41]. Topal and Sinha's experiments were performed with synthetic homopolymer template primers. In this paper we report experiments designed to assess the contribution of the T_4 accessory replication proteins to correct base selection during replication of a natural template: ϕX174 *am*86 template primed with a Taq2 restriction enzyme fragment from wild type ϕX174 DNA.

MATERIALS AND METHODS

Proteins. The gene products (gp) of bacteriophage T_4 genes 32, 41, 43 and 45 were purified according to published procedures [25, 4]

Nucleotides. Unlabelled ribo- and deoxyribonucleotides were supplied as dry powder by Sigma. They were dissolved in a tris-acetate buffer (pH 7.8). Purity was established by chromatography on polyethyleneimine-cellulose (PEI-cellulose) thin layer plates containing a fluorescent indicator (Baker). Lithium chloride was used at 1.0M for pyrmidines and 1.2M for purines as solvent. Radioactive α-^{32}PdTTP was purchased from Amersham-Searle Corporation.

DNA template and primer. Single-stranded DNA from phage ϕX174 mutant am86 was prepared by phenol extraction of phages that had been extensively purified in cesium chloride step and equilibrium density gradients. It was primed for *in vitro* DNA synthesis with a Taq2 restriction enzyme fragment of wild type ϕX174 DNA.

Reaction mix. Generally 30 or 60 μl samples were prepared. In addition to primed template at a concentration of 5.13 μg/ml h, they contained 0.5 mM (pH 7.8), 67 mM potassium acetate, 10 mM magnesium acetate, 1 mM ATP, 0.2 mM each CTP, UTP, GTP and various concentrations of dNTPs indicated in figures and tables. α^{32}P-TTP was used to provide radioactive label. Nuclease-free bovine serum albumin was used at 105μg/ml, the T_4 DNA polymerase (gp43) at 6μg/ml (= 17.4 X excess of gp43/ss DNA), gene 32 protein (gp32) at 112μg/ml, gene 45 protein (gp45) at 12μg/ml, and gene 41 protein (gp41) at 10μg/ml.

Turnover assay. Turnover denotes the template-dependent conversion of precursor deoxynucleotide triphosphate into its monophosphate (dNTP→dNMP). It is a measure of proof-reading by the 3′ to 5′ exonuclease activity of the T_4 DNA polymerase. Reactions were initiated by the reaction mix to 37°. At 0, 10, 20 and 30 min, aliquots (3μl) were removed and applied to PEI-cellulose thin layer plates atop a previously applied mixture of dNTP identical with the labelled nucleotide (in this case α-^{32}P TTP) and the corresponding dNDP and dNMP. Plates were developed by ascending chromatography in 1.0 M lithium chloride [36]. Spots containing mono-, di- and tri-phosphates of thymidine were located by UV absorbance. Polymeric DNA remained at the origin. All the spots including the origin were scraped and counted for distribution of radioactivity in various fractions.

DNA synthesis. This was determined by two independent methods: (1) from the fraction of the radioactive label retained at the origin of PEI-cellulose plates; (2) by spotting aliquots on glass fiber filters and precipitating radio labelled DNA with ice-cold trichloroacetic acid (5%) and 10% saturated sodium pyrophosphate. Filters were washed thrice with 1.0 M HCl and then thrice with 95% ethanol. They were dried and counted in Econofluor (Fisher) scintillation fluid.

RESULTS AND DISCUSSION

Fig. 1 shows the kinetics of DNA synthesis by the T_4 DNA polymerase in the absence of other replication proteins. A wide range of dNTP concentrations present in equimolar quantities was tested. Saturation kinetics were reached at about 200μM.

Effects of individual addition of gene32, gene45 and gene41 proteins on the polymerisation efficiency of the T_4 DNA polymerase were also tested. According to Huberman et al. [17] gene32 protein induces a five-to ten-fold enhancement in the rate of replication of a single-stranded template programmed with polymerase alone. In our experiment gene 32 protein was; used at a concentration of 112μg/ml on the basis of results shown in fig. 3 and 4. As seen 32 protein when the level of precursor dNTPs approaches saturation. The gene 45 protein also induces noticeable increase in the extent of synthesis at higher concentrations of dNTPs (Fig. 5)*

The turnover kinetics are presented in Fig. 6a-6b. When turnover values at 20 min. after the initiation of

* This is consistent with the known role of gene45 protein in enhancing the rate and processivity of DNA synthesis on a primed single stranded template (Mace 1975, Piperno *et al.* 1978).

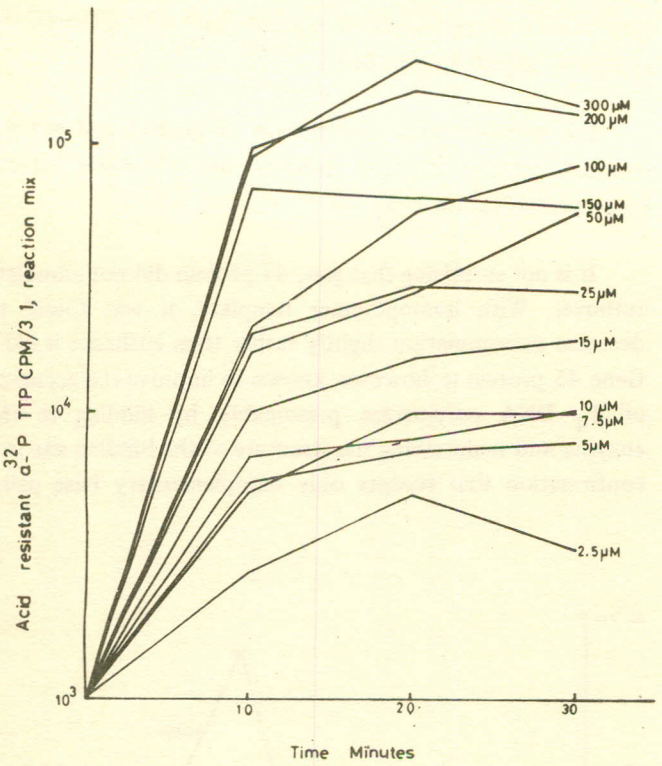

Fig. 1. Kinetics of DNA synthesis by bacteriophage T_4 DNA polymerase. Each point on the curve represents acid resistant radioactive counts in 3µl aliquots at indicated concentrations of dNTPs present in equimolar quantities.

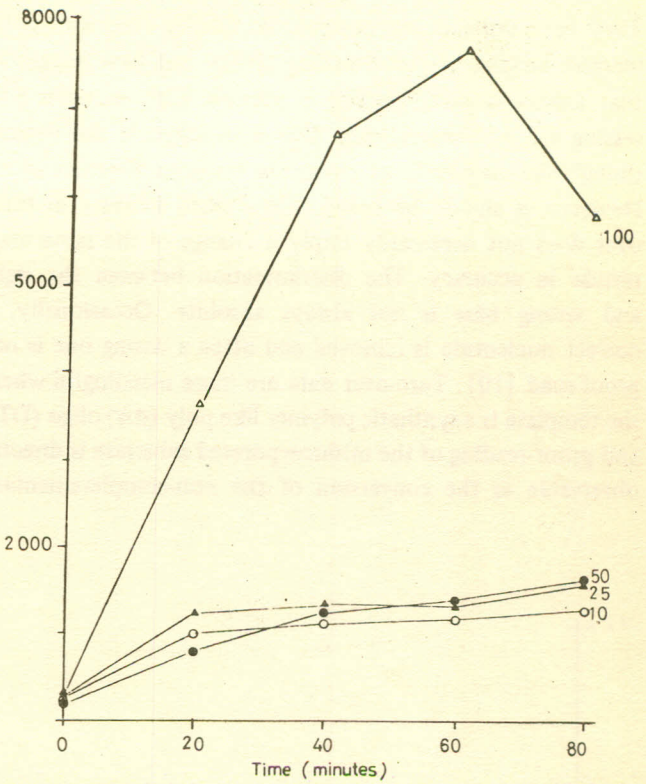

Fig. 2. Kinetics of DNA syntheis in the presence of varying amounts of gene 32 protein (gp32). Each point on the curve represent acid-resistant counts in a 3-µl aliquot of the reaction mix. DNA synthesis was carried out under standard conditions (except for variations in gp32 concentration). Concentrations of gp32 (µg/ml of the reaction mix) are shown against each curve.

synthesis are compared (Fig. 7), it appears that gene32 protein does not stimulate increase in turnover. However, when the values for 10, 20 and 30 min. of syntheses are averaged as shown in Table 1 and replotted as in Fig. 7, gene32 protein appears to cause small increases in turnover.

Topal and Sinha [41] have shown that gp32 interacts with the DNA template to affect the incorporation rather than the proof-reading step of DNA synthesis. It has been suggested that, while hydrogen bonding plays an overriding role for the incorporation of the correctly paired nucleotide, stacking interactions between nearest neighbours dominate in stabilizing the noncomplementary base at the end of the growing chain [40]. It has therefore been inferred that the phage T_4 accessory replication proteins contribute to accuracy by reducing stacking interactions at the end of the growing strand [25, 40].

Kunkel *et al.* [19] have argued that increased fidelity stems from enhanced base selection by the DNA polymerase as a consequence of the increased rigidity of the template due to its association with the single strand binding protein.

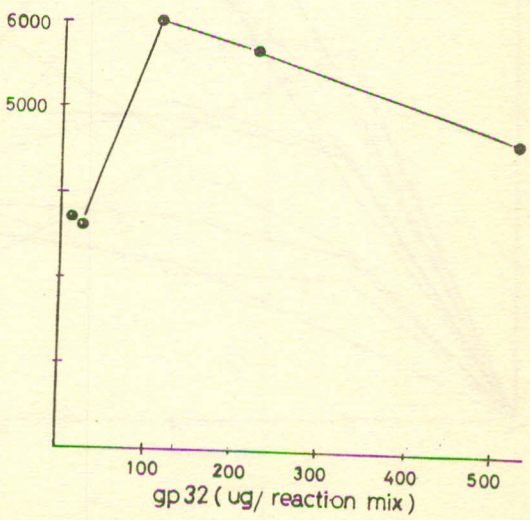

Fig. 3. Effect of variation in gene 32 protein concentration on DNA synthesis. Each point on the curve represent acid-resistant α^{32}P-dATP counts in a 3-µl aliquot of the reaction mix. DNA synthesis was carried out for 60 minutes under standard conditions (except for variations in gp32 concentration).

They have noticed enhancement in fidelity even with polymerases lacking in proof-reading ability but have suggested that enhanced proof-reading is possible with enzymes possessing a 3′→5′ exonuclease. Our data supports this notion, though the magnitude of change in turnover is rather small. However, it should be noted that a small increase in turnover does not necessarily imply a change of the same magnitude in accuracy. The discrimination between the right and wrong base is not always absolute. Occasionally, a correct nucleotide is removed and often a wrong one is not proof-road [10]. Turn-over data are more meaningful where the template is a synthetic polymer like poly (dA) oligo (DT), and proof-reading of the misincorporated substrate is directly observable as the conversion of the non-completementary nucleotide triphosphate to its monphophate e.g. dCTP→dCMP in case of zoly (dA) oligo (dT).

The turnover data in presence of gene45 and gene41 encoded proteins did not indicate any noticeable effects on accuracy (Tables 2–3).

It is not surprising that gene 41 protein did not stimulate turnover. With homopolymer template, it was found to decrease discrimination slightly rather than enthance it (41). Gene 45 protein is, however, known to improve the accuracy of T_4 DNA polymerase presumably by binding to the enzyme and maintaining the structure of the binding site in a confirmation that accepts only complementary base pairs.

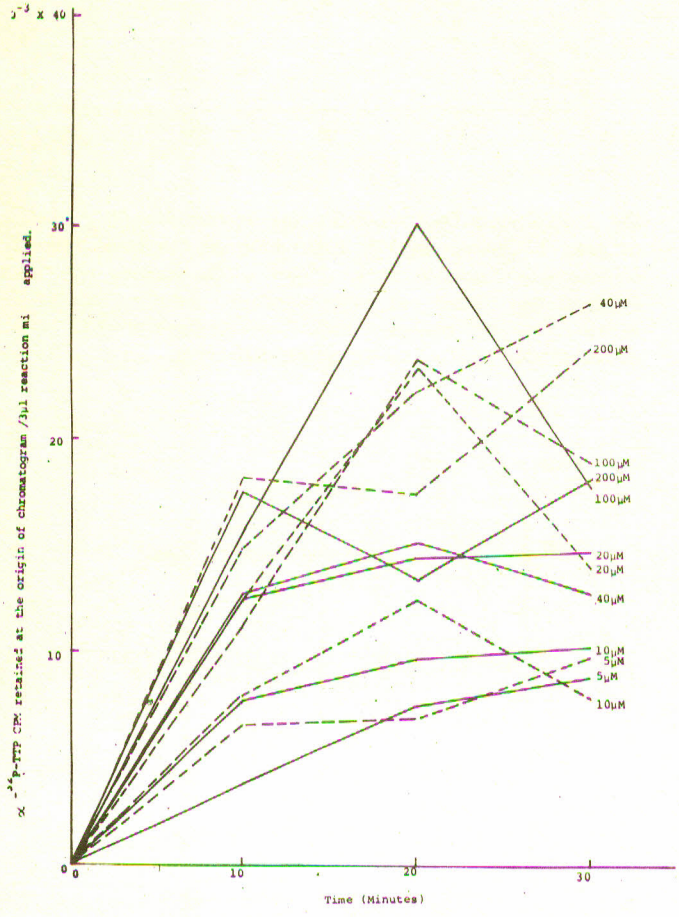

Figure. 4. Kinetics of DNA synthesis with and without gene 32-protein. At the times indicated, 3μl-aliquots were removed and the extent of DNA synthesis determined by chromatography on PEI-cellulose plates as described under "Material and Methods". The four precursor dNTPs were present in equimolar quantities.

gp43(- - - - - -), gp43+gp32 (———)

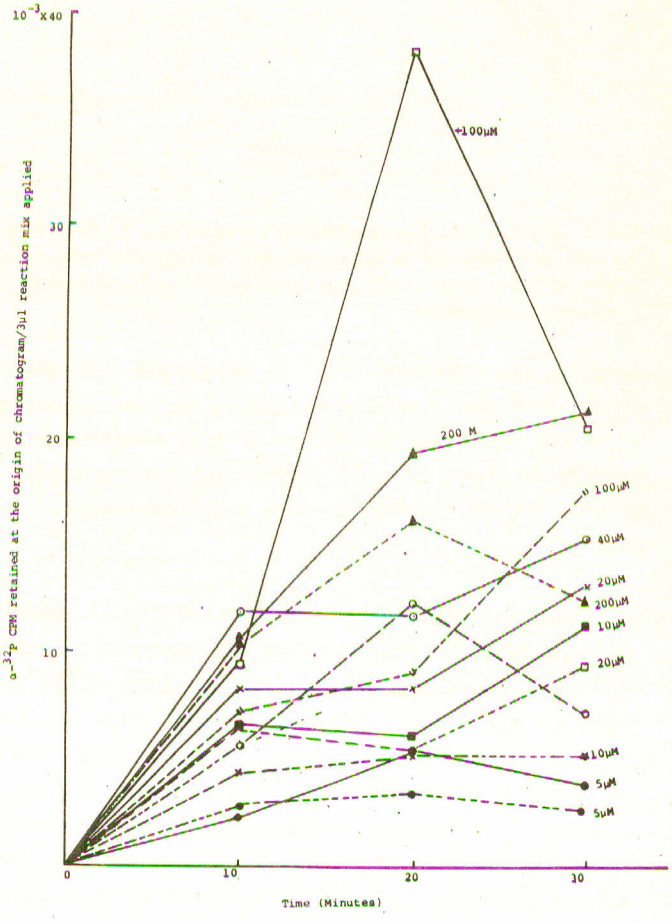

Fig. 5. Kinetics of DNA synthesis by the bacteriophage T_4 DNA polymerase (gp43) with and without the gene45 protein (gp45). At the indicated times, aliquots (3μl) were removed and the extent of DNA synthesis determined by chromatography on PEI-cellulose plates as described under "Materials and Methods". The four precursor dNTPs were present in equimolar quantities. gp43 (- - - - -), gp43+gp45 (——)

Table 1. Effect of gene 32-protein on turnover and stable incorporation of α-^{32}P-TTP labeled precursors by T_4 DNA polymerase during replication of ϕX174 single stranded template

A) POLYMERASE + GENE 32 PROTEIN

1 dNTPs μM each	2 Time after initiation of DNA syntheses	3 % dNMP	4 % DNA	5 % Label used	6 *Turnover*/ Total label used	7 Average/ *Turnover* Total label used
	10 min.	2.46	1.44	3.9	63.08	
5	20 "	3.59	2.52	6.11	58.76	58.98
	30 "	3.82	3.11	6.93	55.12	
	10 "	1.56	1.27	2.83	55.12	
10	20 "	1.9	1.9	3.8	50.00	51.59
	30 "	2.18	2.21	4.39	49.66	
	10 "	1.16	0.85	2.01	57.7	
20	20 "	1.21	1.2	2.41	50.21	52.03
	30 "	1.53	1.58	3.11	49.2	
	10 "	0.58	0.66	1.24	46.77	
40	20 "	0.70	0.72	1.42	49.3	47.08
	30 "	0.80	0.97	1.77	45.2	
	10 "	0.33	0.44	0.77	42.9	
100	20 "	0.35	0.37	0.72	48.61	42.60
	30 "	0.28	0.49	0.77	36.36	
	10 "	0.23	0.17	0.4	57.5	
200	20 "	0.25	0.08	0.33	75.76	62.27
	30 "	0.3	0.26	0.56	53.57	

B) T_4 POLYMERASE ALONE

	10 min.	2.58	3.04	5.62	45.91	
5	20 "	5.80	4.25	10.05	57.71	55.33
	30 "	7.58	4.55	12.13	62.49	
	10 "	0.77	2.07	2.84	27.11	
10	20 "	2.79	2.58	5.37	51.96	40.41
	30 "	1.99	2.82	4.81	41.37	

Contd.

Table 1. (Contd).

1	2	3	4	5	6	7
dNTPs mM each	Time after initiation of DNA syntheses	% dNMP	% DNA	% Label used	Turnover/ Total label used	Average/ Turnover Total label used
	10 "	0.47	1.23	1.7	27.65	
20	20 "	1.57	1.38	2.95	53.22	48.83
	30 "	3.45	1.79	5.24	65.84	
	10 "	0.38	0.82	1.2	31.67	
40	20 "	0.76	0.94	1.7	44.71	35.6
	30 "	0.47	1.16	1.63	28.83	
	10 "	0.63	0.63	1.26	50.00	
100	20 "	1.56	0.50	2.06	75.73	48.72
	30 "	0.18	0.7	0.88	20.45	
	10 "	0.63	0.23	0.86	73.26	
200	20 "	0.96	0.25	1.21	79.34	55.21
	30 "	0.06	0.4	0.46	13.04	

1. This is the concentration of dNTPs in the reaction mixture. Composition of reaction mixture is described under "Materials and Methods". Template is ϕX174 *am*86 single-stranded DNA primed with Taq_2 restriction fragment.
2. These are the time points after initiation of DNA synthesis at which aliquots are removed.
3. This is the percentage of the input α-^{32}P-TTP counts that are scored as dNMP (deoxynucleotide monophosphate).
4. This is the fraction of radioactivity i.e. α-^{32}P-TTP that remains at the origin of chromatogram and represents stable incorporation in DNA.

   Values for both % dNMP and % DNA have been normalized by substracting 'o' minute counts from counts scored at 10, 20 and 30 minutes each.
5. This is obtained by adding % dNMP and % DNA.
6. This is the ratio of counts scored as dNMP to the sum of dNMP+DNA
7. This is obtained by adding Turnover/Total label used values for 10, 20 and 30 minutes and dividing by 3.

Table 2. Turnover and stable incorporation by T_4 DNA polymerase with and without gene 45 protein.

A) T_4 DNA POLYMERASE ALONE

1	2	3	4	5	6
dNTPs μM each	Time after initiation of DNA syntheses	% dNMP	% DNA	% Label used	*Turnover/* Total label used
	0 min.	0.75	1.92	2.67	0.28
	10 "	3.21	6.09	9.3	0.35
5	20 "	4.02	4.99	9.01	0.45
	30 "	5.46	6.53	11.99	0.46

Contd.

Table 2. (Contd).

1 dNTPs μM each	2 Time after initiation of DNA syntheses	3 % dNMP	4 % DNA	5 % Label used	6 *Turnover*/ Total label used
	0 "	0.69	2.02	2.71	0.25
	10 "	1.5	9.51	11.01	0.14
10	20 "	2.36	4.5	6.86	0.34
	30 "	2.27	5.08	7.35	0.31
	0 "	0.65	1.69	2.34	0.28
	10 "	1.19	6.14	7.33	0.16
20	20 "	1.52	4.19	5.71	0.27
	30 "	1.31	5.63	6.94	0.19
	0 "	1.0	1.48	2.48	0.40
	10 "	1.28	7.04	8.32	0.15
40	20 "	1.12	3.83	4.95	0.23
	30 "	0.86	3.46	4.32	0.20
	0 "	0.82	1.47	2.29	0.36
	10 "	0.99	6.03	7.02	0.14
100	20 "	0.98	2.75	3.73	0.26
	30 "	0.54	4.84	5.38	0.10
	0 '.	0.74	1.35	2.09	0.35
	10 "	0.91	5.37	6.28	0.14
200	20 "	0.72	2.71	3.43	0.21
	30 "	0.53	9.92	10.45	0.05
B) DNA POLYMERASE + GENE 45 PROTEIN					
	0 min.	0.72	1.8	2.52	0.29
	10 "	2.09	7.32	9.41	0.22
5	20 "	2.96	5.14	8.1	0.37
	30 "	2.60	9.3	11.9	0.22
	0 "	0.75	1.44	2.19	0.34
	10 "	1.45	4.73	6.18	0.23
10	20 "	2.01	4.96	6.97	0.29
	30 "	2.39	9.62	12.01	0.20
	0 "	1.98	1.45	3.43	0.58
	10 "	1.22	4.52	5.74	0.21
20	20 "	1.38	3.9	5.28	0.26
	30 "	1.01	6.5	7.51	0.13
	0 "	1.7	1.58	3.28	0.52
	10 "	3.33	4.66	7.99	0.42
40	20 "	1.02	3.7	4.72	0.23
	30 "	2.1	5.42	7.52	0.28

Contd.

Table 2 (Contd).

1 dNTPs μM each	2 Time after initiation of DNA syntheses	3 % dNMP	4 % DNA	5 % Label used	6 *Turnover*/ Total label used
	0 "	1.48	1.31	2.79	0.53
	10 "	0.84	4.24	5.08	0.17
100	20 "	0.97	2.45	3.42	0.28
	30 "	0.71	5.02	5.73	0.12
	0 "	0.89	1.38	2.27	0.39
	10 "	0.81	3.09	3.9	0.21
200	20 "	0.78	2.2	2.98	0.26
	30 "	0.52	2.49	3.01	0.17

For explanation of superscript numbers refer to legend for table 1.

Table 3. Turnover and stable incorporation of precursor dNTPs by T_4 DNA polymerase with and without gene 41 protein

(A) DNA POLYMERASE ALONE

1 dNTPs mM each	2 Time after initiation of DNA syntheses	3 % dNMP	4 % DNA	5 % Label used	6 *Turnover*/ Total label used
	0 min.	2.49	1.26	3.75	0.66
	10 "	41.3	6.18	47.48	0.87
2.5	20 "	76.66	5.61	82.27	0.93
	30 "	88.59	5.26	93.85	0.94
	0 "	2.72	0.42	3.14	0.86
	10 "	30.77	7.58	38.35	0.80
5	20 "	64.05	8.6	72.65	0.88
	30 "	80.62	8.7	89.32	0.90
	0 "	2.29	0.89	3.18	0.72
	10 "	1.75	6.9	8.65	0.20
10	20 "	36.2	6.62	42.82	0.85
	30 "	49.67	6.46	56.13	0.88
	0 "	2.511	1.4	3.91	0.64
	10 "	7.11	2.87	9.98	0.71
25	20 "	1.45	3.96	5.41	0.27
	30 "	'3.22	3.77	16.99	0.78
	0 "	2.28	1.6	3.88	0.59
	10 "	4.25	2.2	6.45	0.66
50	20 "	5.87	2.24	8.11	0.72
	30 "	6.75	2.81	9.56	0.71

Contd.

Table 3. (Contd).

1 dNTPs mM each	2 Time after initiation of DNA syntheses	3 % dNMP	4 % DNA	5 % Label used	6 *Turnover*/ Total label used
	0 "	2.42	1.25	3.67	0.66
	10 "	3.62	1.86	5.48	0.66
100	20 "	4.15	1.98	6.13	0.68
	30 "	4.67	1.84	6.51	0.72
	0 "	2.34	1.06	3.4	0.69
	10 "	4.33	1.64	5.97	0.73
200	20 "	3.91	1.38	5.29	0.74
	30 "	3.95	1.34	5.29	0.75
	0 "	2.4	1.21	3.61	0.66
	10 "	3.28	1.37	4.65	0.71
300	20 "	3.12	0.85	3.97	0.79
	30 "	3.05	0.79	3.84	0.79
B) DNA POLYMERASE + GENE 41 PROTEIN					
	0 min.	3.09	2.3	5.39	0.57
	10 "	8.19	12.82	21.01	0.39
2.5	20 "	84.19	7.57	91.76	0.92
	30 "	89.03	5.1	94.13	0.95
	0 "	2.54	1.2	3.74	0.68
	10 "	30.75	7.74	38.49	0.80
5	20 "	63.59	8.59	72.18	0.88
	30 "	79.31	9.35	88.66	0.89
	0 "	2.56	1.21	3.77	0.68
	10 "	15.71	5.45	21.16	0.74
10	20 "	27.37	6.82	34.19	0.80
	30 "	37.82	7.09	44.91	0.84
	0 "	2.52	1.16	3.68	0.68
	10 "	6.1	2.83	8.93	0.68
25	20 "	8.82	2.97	11.79	0.75
	30 "	9.15	3.15	12.3	0.74
	0 "	2.45	1.38	3.83	0.64
	10 "	4.07	2.7	6.77	0.60
50	20 "	5.29	3.02	8.31	0.64
	30 "	7.67	2.83	10.49	0.73
	0 "	2.39	1.26	3.65	0.65
	10 "	3.26	1.88	5.14	0.63
100	20 "	2.57	2.26	4.83	0.53
	30 "	4.66	1.55	6.21	0.75
	0 "	2.66	1.67	4.33	0.61
	10 "	2.84	1.52	4.36	0.65
200	20 "	3.81	0.65	4.46	0.85
	30 "	2.95	1.28	4.23	0.70
	0 "	2.44	1.3	3.74	0.65
	10 "	2.57	1.11	3.68	0.70
300	20 "	2.95	1.06	4.01	0.74
	30 "	3.24	0.8	4.04	0.80

For explanation of superscript numbers refer to the legend of Table 1.

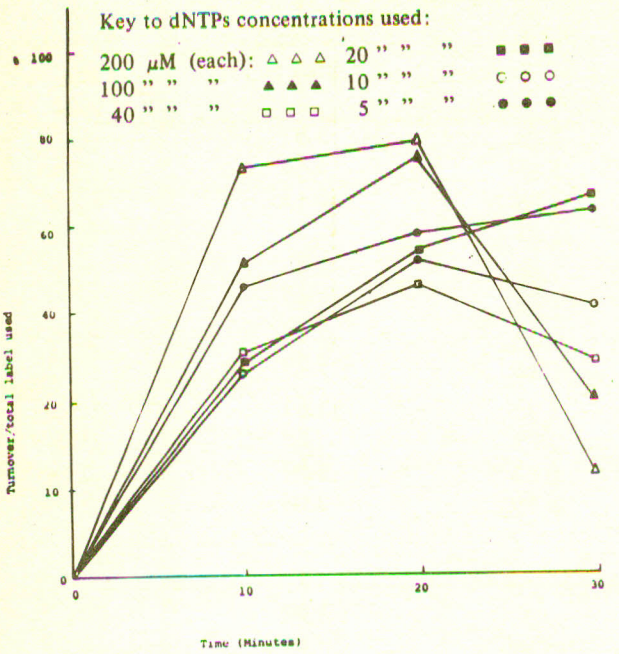

Fig. 6a. Measurement of the level of 3'→5' exonucleases activity of T_4 DNA polymerase in absence of gene 32-protein. Turnover measures DNA-dependent conversion of dNTP → dNMP by the exonuclease. Total label used is the sum of radioactivity incorporated in DNA and that found as dNMP in solution. The precursor nucleotide triphosphates (dATP, dGTP, dCTP and TTP) were present in equimolar quantities. Aliquots (3μl) were removed at indicated times and spotted on PEI-cellulose thin layer plates as described under "Material and Methods". Parallel samples were spotted on glass-fiber filters and the amount of radioactivity in acid-insoluble material determined in order to get an independent assessment of DNA synthesis.

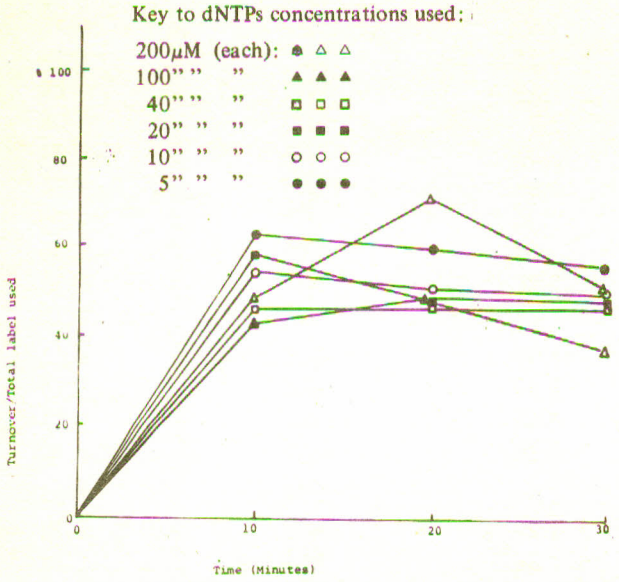

Fig. 6b. Effect of gene 32 protein on 3'→5'. exonuclease activity of T_4 DNA polymerase at various concentrations of dNTPs. Refer to legend for Figure 6a for details.

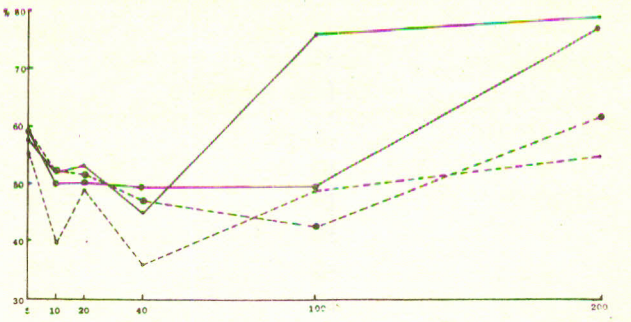

Fig. 7. Effect of gene 32 protein on turnover/total label used at various equimolar concentrations of dNTPs.

% Turnover/total label used 20 minutes after initiation of DNA synthesis.

Polymerase alone (● - ● - ● - ●)

Polymerase + gene 32 protein (● - ● - ● - ●)

% Turnover/otal label used when values for 10,20 and 30 minutes after initiation of DNA synthesis are average.

Polymerase alone (● . . . ● . . . ● . . . ●)

Polymerase + gene 32 protein (● . . . ● . . . ●)

Apparently turnover during replication of a natural template containing all four bases is not a particularly sensitive measure of the role of this protein in modulating fidelity.

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