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The Nucleotide Sequence Adjoining the CCA End of an *Escherichia coli* Tyrosine Transfer Ribonucleic Acid Gene*

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SUMMARY

The sequence of 23 nucleotides beyond the C-C-A end of the *Escherichia coli* tyrosine tRNA gene has been determined. The previously deduced (LOEWEN, P. C., AND KHORANA, H. G. (1973) *J. Biol. Chem.* 248, 3489) sequence of the first 12 nucleotides (T-C-A-A-C-T-T-T-C-A-A-A) has been revised to T-C-A-C-T-T-T-C-A-A-A-A. The sequence of the next 11 nucleotides is G-T-C-C-C-T-G-A-A-C-T. The general approach used in the sequence work was the DNA polymerase-I-catalyzed elongation of suitable deoxyribopolynucleotides which served as primers when hybridized to the r-strand of ϕ80psu$_{III}$ DNA. Five methods were used in the sequential analysis of the elongated primers: (*a*) the use of a restricted number of deoxyribonucleoside triphosphates; (*b*) timed incorporation of all of the four deoxyribonucleoside triphosphates and isolation of the pure radioactive products by polyacrylamide gel electrophoresis; (*c*) isolation of the products from timed incorporation of the dNTPs in which CTP replaced dCTP and Mn^{2+} replaced Mg^{2+}; (*d*) separation of the fragments formed after alkaline hydrolysis by two-dimensional electrophoresis; (*e*) derivation of the nucleotide sequence after partial stepwise degradation by spleen and venom phosphodiesterases by two-dimensional fingerprinting methods of Sanger and co-workers (SANGER, F., DONELSON, J. E., COULSON, A. R., KÖSSEL, H., AND FISCHER, D. (1973) *Proc. Nat. Acad. Sci. U. S. A.* 70, 1209).

The nature of the nucleotide sequences in the DNA regions which determine the initiation and termination of transcription is so far unknown.[1] In connection with the total synthesis of the gene for an *Escherichia coli* tyrosine tRNA, which is in progress in this laboratory, we have initiated studies of the DNA sequences adjoining the two ends of the known nucleotide sequence of the tyrosine tRNA precursor (3, 4). The approach which is being used in this work involves the DNA polymerase-catalyzed elongation of the appropriate deoxyribopolynucleotides which serve as primers when hybridized to the separated strands of the ϕ80psu$_{III}$ DNA. In a previous paper, results were reported of an initial study of the nucleotide sequences beyond the C-C-A end of the tRNA. The sequence of the first 12 nucleotide units was concluded to be T-C-A-A-C-T-T-T-C-A-A-A. This study has now been extended and the sequence of the 23 nucleotides has been deduced.

During this work, it was found necessary to revise slightly the above mentioned sequence of the first 12 nucleotides. The total sequence determined to date is T-C-A-C-T-T-T-C-A-A-A-A-G-T-C-C-C-T-G-A-A-C-T.

The two primers used for hybridization with the r-strand of ϕ80psu$_{III}$ DNA were as shown in Fig. 1. In particular, the second primer which terminated in cytidine (rC) at the 3′ end offered the possibility of selective cleavage to separate the oligonucleotide chain made enzymatically from the original primer. The range of the methods used in sequence work was greater than that used in the previous work (3). The total methods used included the use of a restricted number of deoxyribonucleoside triphosphates, timed incorporation of all the four dNTPs and isolation of homogeneous radioactive products by polyacrylamide gel electrophoresis, preparation of pure elongated primers using CTP in place of dCTP in the enzymatic reactions, alkaline hydrolysis, partial spleen and venom phosphodiesterase degradations and separation of products by two-dimensional electrophoresis and chromatography by the methods of Sanger and co-workers (5, 6).

EXPERIMENTAL PROCEDURE

Materials

These were as described in the previous paper (3) except that the yeast RNA was obtained from Sigma Chemical Co. Cellulose MN 300 and DEAE-cellulose MN 300 were purchased

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[1] Several RNAs transcribed *in vivo* have been shown to terminate in a run of U units followed by 1 A or 2 A units (for a review see Reference 2). Whether this sequence serves as a part of a termination signal remains unknown.

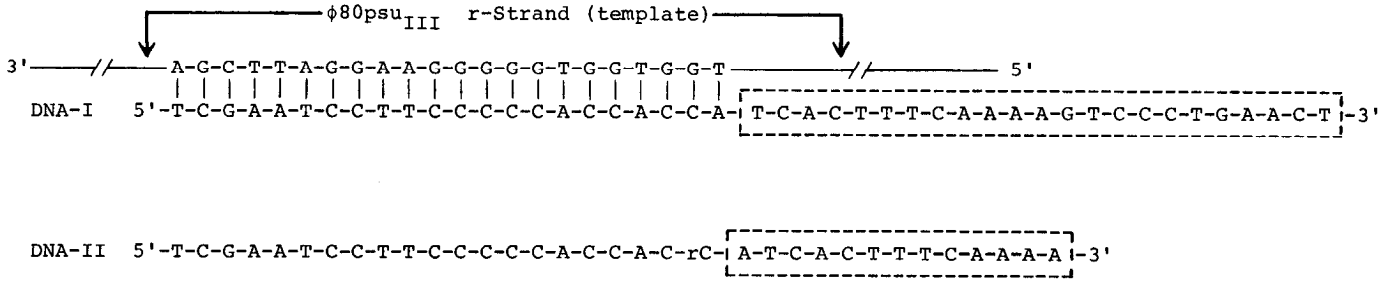

FIG. 1. Diagrammatic representation of the approach to the determination of the nucleotide sequence beyond the 3′ terminus of the tyrosine tRNA gene. The ϕ80psu$_{III}$ r-strand is oriented in the 3′ to 5′ direction from *left* to *right*. Thus, the promoter regions (not shown) will be to the *left* and the terminator region to the *right*. The nucleotide sequences of the two synthetic deoxyribopolynucleotides and their orientation when hybridized to the r-strand are shown. The nucleotide sequences ascertained by the DNA polymerase-I-catalyzed extension of the two primers (DNA-I and DNA-II) are shown in the *dashed boxes*.

from Macherey Nagel and Co. The preparation of DNA-II will be described elsewhere.

Methods

The DNA polymerase-I-catalyzed incorporation of nucleotides, nearest neighbor analysis of the ^{32}P-labeled elongated primers, and the isolation of the pyrimidine tracts after depurination were all carried out as described previously (3).

Isolation of A-T-C-A-C-T-T-T-C-A-A-A-A from Elongated DNA-II

DNA-II was elongated using the reaction conditions described previously (3). The kinetics of incorporation of various deoxyribonucleoside triphosphates were followed as described previously and are shown in the *inset* to Fig. 2. After the reaction, the reaction mixtures labeled with different nucleotides were dialyzed overnight at 5° against 10 mM Tris pH 7.6 and 10 mM EDTA. The solutions were then boiled for 2 min to denature the elongated DNA-II and ϕ80psu$_{III}$ r-strand. After being chilled, the mixtures were then separated by gel filtration through an Agarose 1.5M column (0.9 × 28 cm) pre-equilibrated with 50 mM triethylammonium bicarbonate (pH 7.6). The flow rate was 0.3 ml per 4 min. The elution pattern of the reaction labeled with [α-^{32}P]dATP is shown in Fig. 2. Peak II containing the elongated DNA-II was concentrated and subjected to alkaline hydrolysis for 18 hours with 0.3 N KOH at 37°. The solution was neutralized with acetic acid and subjected to electrophoresis on a strip (3 × 55 cm) of cellulose acetate, pH 3.5, for 45 min at 5000 volts. The autoradiogram of this strip is shown in Fig. 3. The main radioactive spot was eluted with 3 ml of 1 M triethylammonium bicarbonate (pH 7.6) and the solution evaporated to dryness.

Partial Spleen Phosphodiesterase—The partial digestion with the spleen phosphodiesterase was carried out for 30 min at 20° in Pipes (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) sodium salt) buffer (pH 6.9) with 10 mM $MgCl_2$ and 100 μg per ml of the enzyme present.

Partial Venom Phosphodiesterase—The partial digestion with snake venom phosphodiesterase was carried out in glycine buffer, pH 9.2, at 20° for 30 min with 20 μg per ml of the enzyme preparation present.

Two-dimensional Separations—The two-dimensional electrophoretic separation of the alkaline hydrolysis products was carried out following the procedure of Sanger and Brownlee (5). The two-dimensional separation of the partial enzymatic digests using electrophoresis on cellulose acetate (pH 3.5) in the first dimension and homochromatography in the second dimension was as described by Brownlee and Sanger (6).

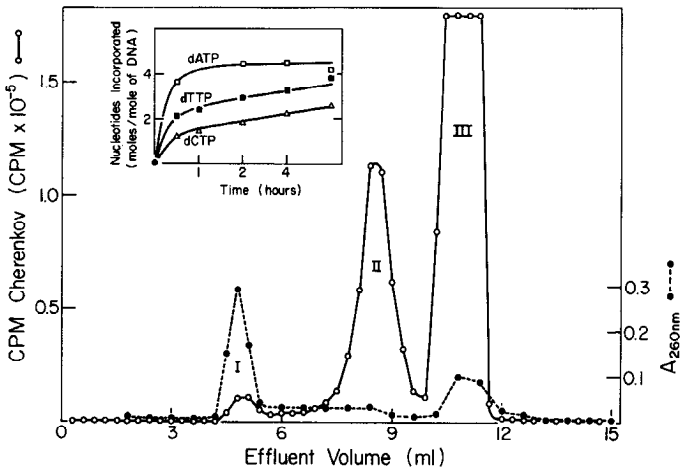

FIG. 2. The kinetics of elongation and column chromatographic separation of the products formed by using DNA-II as the primer. The kinetics of the incorporation of the individual α-^{32}P-labeled deoxyribonucleoside triphosphates in the presence of the other two unlabeled dNTPs are shown in the *inset*. The three deoxyribonucleoside triphosphates, dTTP, dCTP, and dATP were present in all three cases with [α-^{32}P]dCTP being used with dTTP and dATP, △——△; [α-^{32}P]dTTP being used with dCTP and dATP, ■——■; and [α-^{32}P]dATP being used in the presence of dTTP and CTP, □——□. The reaction conditions are described under "Methods." The column fractions were counted Cherenkov, ○——○; and absorbance at 260 nm was determined, ●——●. *Peak I* was the ϕ80psu$_{III}$ r-strand; *Peak II* was the elongated DNA-II; and *Peak III* was excess deoxyribonucleoside triphosphates.

RESULTS

Sequence of First 12 Nucleotides is T-C-A-C-T-T-T-C-A-A-A-A Starting Sequence is T-C-A-C and Not T-C-A-A

The previously reported experiment, which led to the latter sequence, was repeated. DNA-I was elongated by using dTTP or dTTP plus dCTP in the first step. The unused dNTPs were removed and the second step elongations were done using [α-^{32}P]dATP plus dCTP or [α-^{32}P]dATP alone, respectively. The elongated products were then purified by polyacrylamide electrophoresis. This step, which ensured homogeneous products, had not been carried out in the previous work and may account for the discrepancy in the results. The 3′-nucleotide analysis of the products is given in Table I (Experiments 1 and 2). Thus, radioactivity was found predominantly in dCp only. Further, the depurination of both products by the diphenylamine-formic acid procedure gave only pTpC$^*_p$ and no radioactive inorganic

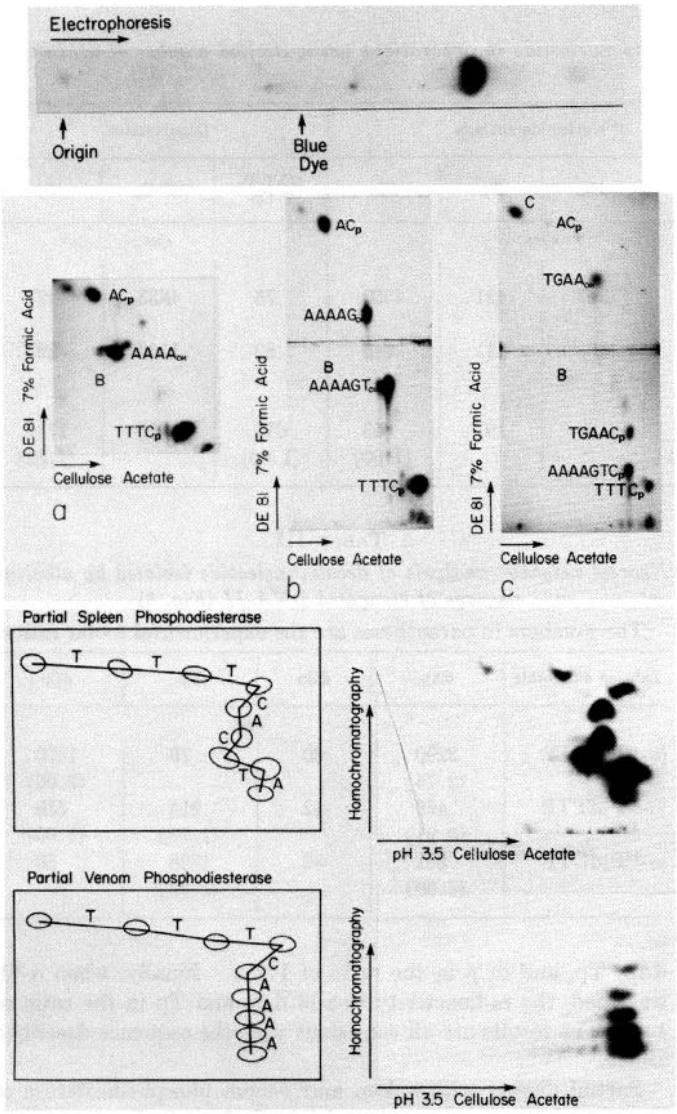

FIG. 3 (*top*). Purification of the elongated portion from DNA-II repair with dTTP, dCTP, and dATP by electrophoresis on cellulose acetate. Elongated DNA-II was isolated as described in Fig. 2 and subjected to alkaline hydrolysis as described under "Methods." The conditions for electrophoresis and elution from cellulose acetate are also described under "Methods."

FIG. 4 (*center*). Two-dimensional paper electrophoretic fingerprint of the fragments from alkaline hydrolysis of various products from the elongation of DNA-I using CTP in place of dCTP. The other dNTPs, dGTP, dATP, and dTTP were present. The elongated products were isolated by gel electrophoresis as described in Fig. 8. After elution from the gel, the products were subjected to alkaline hydrolysis as described previously. The fragments were then subjected to electrophoresis on cellulose acetate pH 3.5 in the first dimension and on DE81 paper in 7% formic acid for the second dimension as described by Sanger and Brownlee (3). The spots were located by autoradiography, eluted, and characterized by partial enzymatic digests and nearest neighbor analyses. The sequence assignment is shown adjacent to each spot. *Fingerprint a* is of the product produced by elongation of DNA-I to 34 nucleotides with dTTP, CTP, and dATP. *Fingerprint b* is of the product R_3, 36 to 37 nucleotides long, isolated as described in Fig. 8. *Fingerprint c* is of the product R_4, 44 to 45 nucleotides long also isolated as in Fig. 8. The letter *B* in each fingerprint marks the location of the blue dye, xylene cyanol FF.

FIG. 5 (*bottom*). Partial enzymatic cleavage of the thirteen nucleotide long fragment isolated from DNA-II after its elongation with dTTP, dATP, and dCTP. The purification of the fragment is described in Fig. 3. The partial enzymatic hydrolysis using spleen phosphodiesterase and snake venom phosphodiesterase are described under "Methods." The digestion products were separated in two dimensions as described under "Methods" and the products located by autoradiography. The fingerprints are shown at the right with that from the partial spleen phosphodiesterase treatment at the *top* and the one from the partial venom phosphodiesterase treatment at the *bottom*. The sequence derivation from the pattern of products is shown at the *left* in both cases. The sequence which can be derived by combining the two partial sequences is A-T-C-A-C-T-T-T-C-A-A-A-A.

phosphate. The latter result is again in contrast with that reported earlier (3).

A-A-A-A Sequence in First 12 Nucleotide Sequence

The following is also a repetition of an earlier experiment. DNA-I was elongated with dTTP and dCTP and the product isolated. Nucleotide additions were next carried out using dCTP and dATP and the product was again separated from the excess of dNTPs. Finally, a repair was carried out with [α-^{32}P]dATP, dCTP, and dTTP. The product was isolated by gel electrophoresis and the major band which corresponded to size 34 was isolated. Nearest neighbor analysis of this product gave a 3:1 ratio of radioactivity in dAp and dCp, while depurination gave a 1:3 ratio of counts in pCTTTC$\overset{*}{\text{p}}$ and $\overset{*}{\text{P}}_{\text{i}}$ (Table I). These results are in agreement with the sequence A-A-A-A_{OH}.

Derivation of Dodecanucleotide Sequence Using dTTP, dATP, and CTP

DNA-I was elongated using dTTP, dATP, and CTP using Mn^{2+} as the divalent cation in the DNA polymerase I-catalyzed reaction. The product was isolated as a single band on polyacrylamide gels and then subjected to alkaline hydrolysis. The resulting fragments were separated by two-dimensional paper electrophoresis and the fingerprint is shown in Fig. 4*a*. Thus, the products found were A-Cp, A-A-A-A_{OH} and T-T-TCp. A-Cp was characterized by 3′-nucleotide analysis (Table II) and by its characteristic electrophoretic mobility on DEAE paper. The fragment A-A-A-A_{OH} was characterized by its size and the number of partial spleen phosphodiesterase degradation products in one-dimensional homochromatography. T-T-TCp was characterized by 3′-nucleotide analysis (Table II) and by the products resulting from partial spleen phosphodiesterase degradation. These results are, thus, consistent with the sequence T-C-A-C-T-T-T-C-A-A-A-A and not T-C-A-A-C-T-T-T-C-A-A-A.

Further evidence supporting the above conclusion comes from analyses of the larger products produced by the elongation of DNA-I with CTP, dATP, dGTP, and dTTP. While detailed characterization of the products and discussion of the fragments obtained on alkaline hydrolysis are given later, of interest from the present standpoint were the fragments A-A-A-A-G_{OH}, A-A-A-A-G-T_{OH}, and A-A-A-A-G-T-Cp. The nearest neighbor analyses for these fragments (Table II) are all consistent with the new sequence assignment.

Derivation of Dodecanucleotide Sequence Using DNA-II and dTTP, dCTP, and dATP

DNA-II containing rC at the 3′ terminus was elongated with dTTP, dCTP, and dATP one of which was labeled at a time by ^{32}P at the α position. The products were isolated away from the template DNA and the excess of dNTPs by gel filtration (Fig. 2) through columns of Agarose 1.5 M and subjected to alkaline hydrolysis. The newly formed radioactive segments thus obtained were purified by cellulose acetate electrophoresis (Fig. 3).

TABLE I

Characterization of elongated products formed from DNA-I by two-step nucleotide incorporations using limited number of dNTPs

The numbers in parentheses are the experimental molar ratios.

Experiment	Step	Nucleotides present	3′-Nucleotide analysis				Depurination		
			dAp	dGp	Tp	dCp	pC-T-T-T-Cp	pT-Cp	P_i
			cpm				*cpm*		
1	1	dTTP							
	2	[α-^{32}P]dATP + dCTP	602	367	491	4250	76	4833	477
2	1	dTTP + dCTP							
	2	[α-^{32}P]dATP	178	129	111	2042	89	1166	267
3	1	dTTP + dCTP							
	2	dCTP + dATP							
	3	[α-^{32}P]dATP + dCTP + dTTP	1517 (2.73)	129	197	553 (1.00)	615 (1.00)	65	1734 (2.82)

TABLE II

Nearest neighbor analysis of fragments formed after alkaline hydrolysis of elongated DNA-I (Fig. 4)

For isolation of Bands R_3 and R_4 see the legend to Fig. 8.

Fragment	Label	dAp	dGp	Tp	Cp
DNA-I elongated with dTTP, dATP, and CTP					
A-Cp	[α-^{32}P]dTTP	16	21	33	278
	[α-^{32}P]CTP	351	33	29	21
	3[α-^{32}P]NTP	281	27	25	250
T-T-T-Cp	[α-^{32}P]dTTP	83	69	1230	32
	[α-^{32}P]CTP	42	44	277	54
	[α-^{32}P]dATP	41	59	21	602
	3[α-^{32}P]NTP	42	34	831	310
A-A-A-A_{OH}	[α-^{32}P]dATP	1107	57	63	34
	3[α-^{32}P]NTP	573	43	37	51
Band R_3 of Fig. 8					
A-A-A-A-G_{OH}	[α-^{32}P]dATP	249	58	11	9
	[α-^{32}P]dGTP	133	36	43	31
	4[α-^{32}P]NTP	923	67	26	32
A-A-A-A-G-T_{OH}	[α-^{32}P]dATP	1364	62	18	25
	[α-^{32}P]dGTP	470	28	16	8
	[α-^{32}P]dTTP	33	456	52	27
	4[α-^{32}P]NTP	926	208	20	35
Band R_4 of Fig. 8					
A-A-A-A-G-T-Cp	[α-^{32}P]dATP	1101	55	18	25
	[α-^{32}P]dGTP	840	26	30	5
	[α-^{32}P]dTTP	76	339	58	22
	[α-^{32}P]CTP	34	65	335	309
	4[α-^{32}P]NTP	997	297	278	206
T-G-A-A	[α-^{32}P]dATP	464	439	26	14
	[α-^{32}P]dGTP	11	35	498	13
	4[α-^{32}P]NTP	323	286	321	15
T-G-A-A-Cp	[α-^{32}P]dATP	711	674	33	15
	[α-^{32}P]dGTP	18	14	338	4
	[α-^{32}P]dTTP	35	9	20	790
	[α-^{32}P]CTP	850	47	57	96
	4[α-^{32}P]NTP	246	123	120	94

Nearest neighbor analysis of the products gave the results shown in Table III. As is seen, when [α-^{32}P]dATP was used, radioactivity was found in dAp and dCp in the ratio of 3:2. When [α-^{32}P]dTTP was used the radioactivity was found in dAp, Tp, and dCp in the ratio of 1:2:1. Finally, when α-^{32}P was used, the radioactivity was in dAp and Tp in the ratio of 1:2. The results are all consistent with the sequence described above.

TABLE III

Nearest neighbor analysis of decatrinucleotide isolated by alkaline cleavage of elongated DNA-II (Fig. 3)

The numbers in parentheses are the experimental molar ratios.

Labeled nucleotide	dAp	dGp	Tp	dCp
	cpm			
[α-^{32}P]dATP	2290 (2.74)	60	79	1690 (2.00)
[α-^{32}P]dTTP	466 (0.91)	42	914 (1.79)	510 (1.00)
[α-^{32}P]dCTP	891 (1.00)	32	1728 (1.93)	50

Partial digests with spleen and venom phosphodiesterase of the labeled elongated segment obtained after alkaline hydrolysis were separated in two dimensions using cellulose acetate ionophoresis and homochromatography. The fingerprint of the products from the partial spleen digestion is shown in Fig. 5 and the shifts of each succeeding fragment reveal the 5′ end sequence as A-T-C-A-C-T-T-T-. . . . The fingerprint of the products from the partial venom digestion is also shown in Fig. 5 and the sequence at the 3′ end thus is . . . T-T-T-C-A-A-A-A. Therefore, the total results using DNA-II provide further confirmation of the revised sequence.

Sequence of Next 11 Nucleotides is G-T-C-C-C-T-G-A-A-C-T

Derivation of Sequence by Stepwise Nucleotide Additions

First Nucleotide Adjoining A-A-A-A is G—The presence of G in the thirteenth position was inferred from the kinetic experiments already reported (3). Thus, a plateau in incorporation was reached after a chain of 12 nucleotide units when the three triphosphates, dATP, dCTP, and dTTP were used. When dGTP was also included, the incorporation was much more extensive and prolonged (3). In a more controlled experiment, the primer, DNA-I was elongated with unlabeled dTTP, dCTP, and dATP and the resulting product plus r-strand template complex was isolated by gel filtration and dialysis against the buffer used in DNA polymerase reactions. Nucleotide incorporation was next carried out using [α-^{32}P]dGTP alone. As

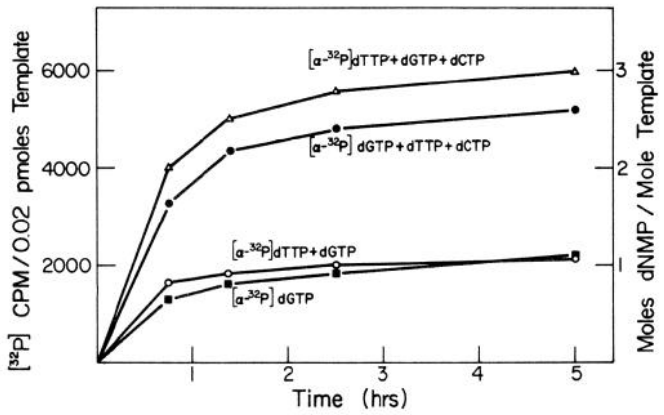

FIG. 6. Kinetics of the repair of DNA-I with α-^{32}P-labeled deoxyribonucleoside triphosphates. DNA-I was first elongated with unlabeled dTTP, dCTP, and dATP. The DNA-primer duplex was isolated away from the excess dNTPs as described previously (2). A second set of repair reactions was then carried out using [α-^{32}P]dGTP, ■——■; [α-^{32}P]dTTP + dGTP, ○——○; [α-^{32}P]dGTP + dCTP + dTTP, ●——●; and [α-^{32}P]dTTP + dGTP + dCTP, △——△. The conditions are described under "Methods."

seen in Fig. 6, 1 mole of this nucleotide per mole of the template was incorporated. After separation from the labeled triphosphate, the product was purified by electrophoresis on 12% polyacrylamide gel under denaturing conditions. One predominant band, corresponding to about 36 nucleotides long, was found (Fig. 7). Nearest neighbor analysis of this material gave radioactivity primarily in dAp (Table IV), although fairly high background counts in other mononucleotides were observed. Thus, only 1 dGMP unit had been added to the terminal A unit (the twelfth nucleotide). Independent evidence for this conclusion is given below using all four deoxyribonucleoside triphosphates.

Nucleotide Next to G is T—Elongation of DNA-I using cold dTTP, dCTP, and dATP was repeated but after isolation of the elongated primer plus r-strand complex, second step elongation was carried out with dGTP and [α-^{32}P]dTTP. As seen in Fig. 6, 1 mole of the labeled nucleotide was incorporated per mole of the template DNA. The product was again isolated and purified as described above, one main band of approximately the same size as the preceding product being found (Fig. 7*c*). The 3′-nucleotide analysis gave predominantly radioactivity in dGp showing the sequence -G-T (Table IV). Depurination of

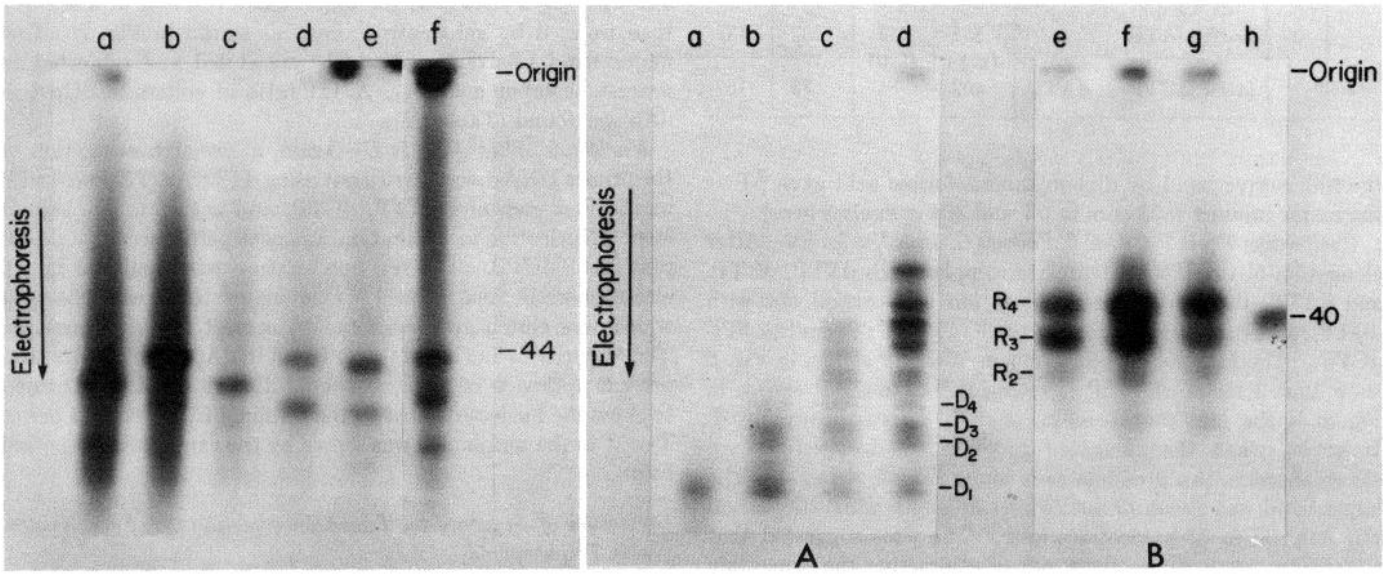

FIG. 7 (*left*). Gel electrophoresis of various chain elongated products. Products from the stepwise elongation of DNA-I were separated from the excess dNTPs as described under "Methods," denatured in 100% formamide and subjected to electrophoresis on a 12% polyacrylamide gel in 7 M urea. DNA-I had been elongated with dTTP, dCTP, and dATP and isolated for further repair reactions as described in Fig. 6. *Channel a* contained this DNA elongated with [α-^{32}P]dGTP. *Channel b* contained this DNA elongated with [α-^{32}P]dGTP + dCTP + dTTP. *Channel c* contained this DNA elongated with [α-^{32}P]-dTTP + dGTP. *Channel d* contained this DNA elongated with [α-^{32}P]dTTP + dGTP + dCTP. *Channel e* contained this DNA elongated with [α-^{32}P]dCTP + dTTP + dGTP. In *Channel f*, DNA-I was first elongated with unlabeled dATP, dCTP, and dTTP, and subsequently with dGTP, dTTP, and dCTP. After isolation from the excess dNTPs, this DNA was then elongated with [α-^{32}P]dATP. The number 44 at the right denotes the location on the gel of a 44 nucleotide long oligonucleotide. The bands were eluted and are characterized in Table IV.

FIG. 8 (*right*). Gel electrophoresis of the elongated products resulting from the repair of DNA-I in the presence of all four dNTPs or three dNTPs and CTP for various lengths of time. The reaction conditions were as described under "Methods" except that Pipes buffer pH 6.9 replaced phosphate buffer in the reactions with CTP (Fig. 8*A*). A reaction mixture of 200 μl containing 2 pmoles of r-strand, 6 pmoles of DNA-I, [α-^{32}P]dCTP, dTTP, dGTP, and dATP was prepared at 5°. Aliquots of 50 μl were removed at 8 min (*Channel a*), 15 min (*Channel b*), 30 min (*Channel c*), and 60 min (*Channel d*), quenched with 20 mM EDTA, desalted as described previously (2), denatured in 100% formamide, and subjected to electrophoresis on a 12% polyacrylamide gel containing 7 M urea (Fig. 8*B*). A similar reaction mixture, but containing 25 μM CTP in place of dCTP, 0.5 mM $MnCl_2$ in place of $MgCl_2$, [α-^{32}P]dATP, dGTP, and dTTP was prepared. Aliquots were removed at 30 min (*Channel e*), 60 min (*Channel f*), and 120 min (*Channel g*) treated as described above and subjected to electrophoresis on a 15% polyacrylamide gel containing 7 M urea. The bands were localized by autoradiography and eluted with 1 M triethylammonium bicarbonate for characterization. *Channel h* contained a 38 to 40 duplex from the tyrosine tRNA precursor [*B*] part (7) as a marker on the 15% gel. The letters D_1, D_2, D_3, and D_4 denote various bands from the 12% gel containing all four dNTPs. The letters R_2, R_3, and R_4 denote the bands from the experiment containing CTP.

TABLE IV

Nearest neighbor analysis of elongated products formed from DNA-I by two step nucleotide incorporations using limited number of dNTPs (Fig. 7)

The numbers in parentheses are the experimental molar ratios.

Experiment	Step	Nucleotides present	dAp	dGp	Tp	dCp
			cpm			
1	1	dTTP + dATP + dCTP				
	2	[α-^{32}P]dGTP	1050	150	201	166
		or				
		[α-^{32}P]dTTP + dGTP	209	720	171	86
		or				
		[α-^{32}P]dTTP + dGTP + dCTP	158	557 (1.0)	145	594 (1.1)
		or				
		[α-^{32}P]dCTP + dTTP + dGTP	129	617	1393 (1.0)	3431 (2.4)
		or				
		[α-^{32}P]dGTP + dTTP + dCTP	1121 (1.0)	514	1245 (1.1)	519
2	1	dTTP + dATP + dCTP				
	2	dTTP + dGTP + dCTP				
	3	[α-^{32}P]dATP	551 (1.1)	516 (1.0)	102	137
		or				
		[α-^{32}P]dCTP + dATP	402	64	73	152

the radioactive band by diphenylamine-formic acid gave $\overset{*}{p}$T as the major product (853 cpm in pT and 304 cpm elsewhere).

Pyrimidine Tract T-C-C-C-T Follows G, 13th Nucleotide—After elongation of the DNA-I-template complex with dTTP, dCTP, and dATP, the second stage elongation was carried out with dGTP, dCTP, and [α-^{32}P]dTTP or dGTP, dTTP, and [α-^{32}P]-dCTP. The kinetics of incorporation of dTTP shown in Fig. 6 show that 3 moles of TMP were incorporated. Not shown in Fig. 6 is the fact that 5 moles of dCMP were incorporated. However, when the elongated product was isolated by gel electrophoresis, two products were found (Fig. 7, *d* and *e*). The larger band was about 40 nucleotides in length while the second was only 30 to 32 nucleotides long. The sizes suggested that the shorter product was the result of completing the elongation of an incompletely elongated product from the first stage of nucleotide incorporation. The larger bands obtained using either [α-^{32}P]dCTP or [α-^{32}P]dTTP as the radioactive triphosphates were isolated from the gels and characterized.

The nearest neighbor analysis of the [^{32}P]TMP-labeled product gave a 1:1 ratio of counts in dGp and dCp (557 cpm in dGp and 594 cpm in dCp; Table IV). The [^{32}P]dCMP-labeled product gave a 1:2.4 ratio of counts in dCp and dTp (3431 cpm in dCp and 1393 cpm in Tp; Table IV). Upon depurination the products gave one pyrimidine tract containing radioactivity. When the ^{32}P label was in dpT, the pyrimidine tract was 50% phosphatase-sensitive and nearest neighbor analysis of the dephosphorylated product gave all of the radioactivity in dCp. When the label was in dpC the ^{32}P label in the pyrimidine tract was phosphatase-insensitive but the dephosphorylated product had the same mobility as the dephosphorylated product labeled with TMP. Nearest neighbor analysis of this tract labeled with dpC gave a 1:2 ratio of the radioactivity in Tp and dCp, respectively (194 cpm in Tp and 378 cpm in dCp). Hence, the sequence of the pyrimidine tract could unambiguously be deduced as T-C-C-C-T. From its mobility, both before and after dephosphorylation, it was concluded that the product was phosphorylated at both ends indicating a G residue at the 3′ end as well.

Nucleotide Next to G-T-C-C-C-T Is G—This has already been concluded above. Further evidence was obtained by the DNA-I elongation with dTTP, dCTP, and dATP followed by a second step elongation using dTTP, dCTP, and [α-^{32}P]dGTP. The major product isolated after gel electrophoresis (Fig. 7*b*) was 40 nucleotide units long and gave, on degradation to 3′-nucleotides, a 1:1 ratio of radioactivity in dAp and Tp (1121 cpm in dAp and 1245 cpm in Tp; Table IV). Thus, it was concluded that a G unit followed the pyrimidine tract.

Next Two Nucleotides are A-A; Thus, Sequence Is G-T-C-C-C-T-G-A-A—The above described two-step elongation of the primer DNA-I (dTTP, dCTP, and dGTP at the second stage) reached a plateau at the sequence G-T-C-C-C-T-G. This result implied that the next nucleotide must be A. The experiment to prove this was similar to those above described. The primer DNA-I was elongated with unlabeled dTTP, dATP, and dCTP followed by separation from the excess of dNTPs. A subsequent synthesis with unlabeled dGTP, dCTP, and dTTP was then carried out followed by isolation as before. Finally, elongation was carried out with [α-^{32}P]dATP as the label. The reaction product was then isolated by gel electrophoresis as shown in Fig. 7*f*. One major band was observed which was eluted and subjected to nearest neighbor analysis. A 1:1 ratio of counts in dGp and dAp was found (Table IV).

Nucleotide After A-A Is C—Again, a two-step elongation of the primer DNA-I was carried out using dTTP, dCTP, and dATP in the first step and dTTP, dCTP, and dGTP in the second step. Nucleotide incorporation was next performed with dATP plus [α-^{32}P]dCTP. The reaction mixture was separated by gel electrophoresis (not shown). One major band was observed which was eluted and subjected to nearest neighbor analysis. The majority of the counts were found in dAp (Table IV). The present approach of using restricted dNTPs could not be used to show the nucleotide next to the terminal C. The presence of T next to the terminal C was shown by the experiments reported below.

Derivation of Sequence by Timed Incorporation of Four Nucleoside Triphosphates

Two experiments were carried out, one in which only the deoxyribonucleoside triphosphates were used and the second in which CTP replaced dCTP. The reaction mixtures were set up as in the legend to Fig. 8, and aliquots removed at various times were examined by electrophoresis. The results of electrophoresis on 12% or 15% polyacrylamide gels are shown in Fig. 8. The size of each of the products formed was estimated by comparison of its mobility with those of synthetic deoxyribopolynucleotides of known length (Fig. 9). The structures of the major products formed in the two experiments are given in Fig. 10.

Products Formed Using CTP, dTTP, dATP, and dGTP—In the experiment shown in Fig. 8*B*, the labeled nucleotide was [α-^{32}P]dATP. As is seen, two major products, designated R_3 and R_4, were formed (the minor products are designated R_1 and R_2). The size of R_3 was determined to be 37 ± 2 (Fig. 9), while R_4 was found to be 44 ± 2 nucleotides.

Product R_3 (Figs. 8 to 10)—The size of this product determined from its mobility was consistent with the structure shown in

Fig. 10. The results of nearest neighbor analyses using different α-^{32}P-labeled dNTPs or [α-^{32}P]CTP are shown in Table V. All the transfers are in agreement with the structure in Fig. 10 except for the partial transfer from [^{32}P]pT to dGp. This result may indicate that the product R_3 is heterogeneous, one part terminating in dG and another part in T at the 3′ end. The heterogeneity would explain the broadness of the R_3 band on the gel.

In another experiment, the product R_3, prepared by using all the four [α-^{32}P]nucleoside triphosphates, was subjected to alkaline hydrolysis and the resulting fragments were separated by electrophoresis in two dimensions. The fingerprint obtained is shown in Fig. 4*b*. Four main spots were obtained and their characterization by nearest neighbor analysis and partial enzymatic degradation are shown in Table II. Similar experiments carried out using individual [α-^{32}P]dNTPs gave results which are included in Table II. As is seen, the expected fragments were found.

As seen in Fig. 4*b* and Table II, the product R_3 contained A-Cp, T-T-T-Cp, and two more spots which were identified as A-A-A-A-G_{OH} and A-A-A-A-G-T_{OH} (Tables II and VI and partial spleen and venom phosphodiesterase degradations). Clearly, the latter two spots represented elongation of A-A-A-A_{OH} which is seen in Fig. 4*a*.

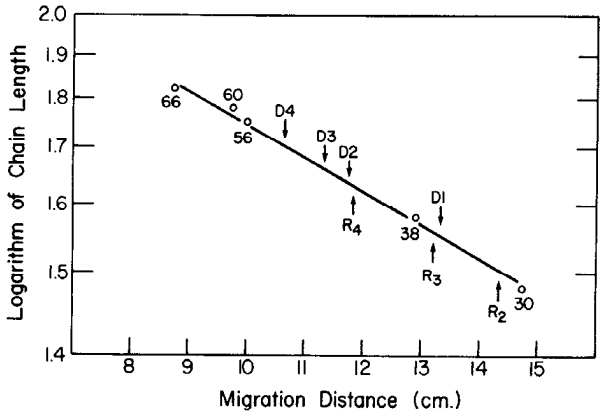

Fig. 9. Size determination of the various Fragments D_1 to D_4 and R_2 to R_4 isolated from the elongation of DNA-I for various times. Deoxyribopolynucleotide fragments from the tyrosine tRNA precursor molecule (7) were subjected to electrophoresis on a 12% gel along side the various elongation products. The migration distances of the tRNA gene fragments in the gel were plotted against the logarithm of their known chain length. The various elongated products were positioned according to their migration distance and their approximate chain length determined.

As expected, when R_3 was labeled with [^{32}P]dAMP, the fragment A-Cp was absent from the fingerprint. Similarly, A-A-A-A-G_{OH} was absent when R_3 was labeled with [^{32}P]TMP and both A-A-A-A-G_{OH} and A-A-A-A-G-T_{OH} were absent from the product labeled with [^{32}P]CMP.

The presence of both A-A-A-A-G_{OH} and A-A-A-A-G-T_{OH} in the fingerprint of R_3 confirms that there is a tetra-A sequence in

TABLE V

Nearest neighbor analysis of DNA-I elongated with CTP and 3 dNTPs (Fig. 8)

The numbers in parentheses are the experimental molar ratios.

Labeled nucleotide	dAp	dGp	Tp	Cp
	cpm			
Band R_3				
[α-^{32}P]dTTP	390 (1.1)	238 (0.6)	677 (1.9)	362 (1.0)
[α-^{32}P]CTP	1403 (1.0)	275	2392 (1.7)	327
[α-^{32}P]dATP	2347 (2.9)	257	174	1600 (2.0)
[α-^{32}P]dGTP	1081 (1.0)	369	518	329
Band R_4				
[α-^{32}P]dTTP	624 (1.4)	346 (0.8)	946 (2.0)	807 (1.8)
[α-^{32}P]CTP	362 (1.5)	144	628 (2.7)	491 (2.1)
[α-^{32}P]dATP	1301 (3.9)	336 (1.0)	190	605 (1.8)
[α-^{32}P]dGTP	1067 (1.0)	416	1037 (1.0)	502

TABLE VI

5′-Nucleotide analysis of fragments after alkaline hydrolysis of elongated DNA-I (Fig. 4)

The numbers in parentheses are the experimental molar ratios.

Fragment	dpA	dpG	pT	dpC
	cpm			
A-A-A-A-G	473 (3.2)	147 (1.0)	44	63
A-A-A-A-G-T	736 (2.6)	282 (1.0)	185 (0.7)	15
T-G-A-A	2424 (1.8)	1367 (1.0)	39	20

PRODUCT D_1 5′-T-C-G-A-A-T-C-C-T-T-C-C-C-C-C-A-C-C-A-C-C-A-T-C-A-C-T-T-T-C-A-A-A-A-G -3′

PRODUCTS D_2, D_3, D_4 5′-T-C-G-A-A-T-C-C-T-T-C-C-C-C-C-A-C-C-A-C-C-A-T-C-A-C-T-T-T-C-A-A-A-A-G-T-C-C-C-T-G-A-A-C-T--3′

PRODUCT R_3 5′-T-C-G-A-A-T-C-C-T-T-C-C-C-C-C-A-C-C-A-C-C-A-T-rC-A-rC-T-T-T-rC-A-A-A-A-G-T -3′

PRODUCT R_4 5′-T-C-G-A-A-T-C-C-T-T-C-C-C-C-C-A-C-C-A-C-C-A-T-rC-A-rC-T-T-T-rC-A-A-A-A-G-T-rC-rC-rC-T-G-A-A-rC-T--3′

Fig. 10. Sequences of the repair products from timed incorporation experiments. The product Bands D_1 to D_4, R_3 and R_4 were isolated as described in the legend to Fig. 8 and their size was determined as described in Fig. 9. The sequence derivation is described under "Results." The primer DNA-I is at the *left* in *small letters* and the elongated portions are shown in *boldface letters*.

the first twelve nucleotides and that a single G residue follows this sequence.

Product R_4 (Figs. 8 to 10)—The size 44 ± 2 indicated for R_4 suggested that it corresponded closely to the sequence determined by stepwise elongations. The nearest neighbor analyses of the samples of the product R_4 as given in Table V are, in general, consistent with the structure shown in Fig. 10, although there are high backgrounds and deviations from the expected distribution of radioactivity. For further work, R_4 was prepared using all the four [α-^{32}P]NTPs and then subjected to alkaline hydrolysis. The resulting fingerprint is shown in Fig. 4*c*. Thus, as expected, A-Cp and T-T-T-Cp were present, but there were also three new major spots in addition to a few minor ones. One fragment was characterized to be A-A-A-A-G-T-Cp by nearest neighbor analysis (Table II) and partial spleen phosphodiesterase treatment. The fragment A-A-A-A-G-T_{OH} from R_3 had, therefore, been converted to A-A-A-A-G-T-Cp, confirming the sequence of the three nucleotides following the A-A-A-A sequence. The two other new spots were identified as T-G-A-A_{OH} and T-G-A-A-Cp. The nearest neighbor analyses are given in Table II, and the 5′-nucleotide analysis of T-G-A-A is given in Table VI. Further, the size of the fragment as determined by homochromatography and the number of products formed from partial spleen phosphodiesterase treatment were all consistent with the above structures.

The above evidence supported the structure of R_4 as shown in Fig. 10, but also showed that it was admixed with the homolog lacking the terminal C-T sequence.

Further proof for these conclusions was obtained by preparing R_4 labeled with individual [α-^{32}P]nucleoside triphosphates. As expected, in the fingerprint prepared from R_4 labeled with [^{32}P]-TMP or [^{32}P]CMP, the fragment T-G-A-A_{OH} was missing, while the fragment T-G-A-A-Cp was present. The fragment from R_4 labeled with [^{32}P]CMP gave transfer only to dAp (Table II), while the same fragment labeled with [^{32}P]TMP gave transfer only to Cp. This result showed that there is a T residue adjacent to the final C residue.

Products Formed Using dCTP, dTTP, dATP, and dGTP (Fig. 8A)

Product D_1—The size estimate for this product indicated it to be in the neighborhood of 35 nucleotides. Nearest neighbor analysis of the product labeled in individual reactions with one of the four [α-^{32}P]deoxyribonucleoside triphosphates are shown in Table VII. Indeed, none of the labeled products gave a ^{32}P transfer to dGp, suggesting that D_1 terminates in a G residue. The nucleotide transfers were all consistent with the sequence and size shown in Fig. 10. Furthermore, depurination of the product samples prepared by using labeled dTTP, dCTP, or dATP resulted in only the two pyrimidine tracts pC-T-T-T-Cp and pT-Cp.

Products D_2, D_3, and D_4—The size of these products, 45, 47, and 52 nucleotides, respectively, indicated that they were equal to or longer than the sequence of 23 nucleotides determined by the stepwise elongation of DNA-I. The nearest neighbor analysis of labeled products D_2 and D_3 are given in Table VII. The transfer for D_2 and D_3 are very similar, except for a partial pT to dCp transfer. Since the bands are very close together on the gels, there are two possible explanations. The first is that there actually is only one nucleotide difference between D_2 and D_3, or, secondly, that during the cutting of the gel some mixing of the two bands occurred. However, the transfers in both cases are consistent with the sequence derived by stepwise elongation including an additional two or three nucleotides at the 3′ end. There are additional transfers, dpC to dCp and dpC to Tp as well as dpG to dCp, which could suggest an additional sequence (T)-C-C-G. There is also an additional pT to dAp transfer which cannot be accounted for readily.

TABLE VII

Nearest neighbor analysis of DNA-I elongated with all four dNTPs (Fig. 8)

The numbers in parentheses are the experimental molar ratios.

Labeled nucleotide	dAp	dGp	Tp	dCp
Band D_1				
[α-^{32}P]dTTP	723 (1.1)	158	1163 (1.8)	657 (1.0)
[α-^{32}P]dCTP	566 (1.0)	90	1129 (2.0)	90
[α-^{32}P]dATP	6551 (3.1)	288	464	4162 (2.0)
[α-^{32}P]dGTP	360 (1.0)	109	72	60
Band D_2				
[α-^{32}P]dTTP	512 (1.9)	275 (1.0)	509 (1.9)	309 (1.4)
[α-^{32}P]dCTP	211 (1.8)	96	477 (4.0)	343 (2.8)
[α-^{32}P]dATP	4162 (4.5)	878 (1.0)	619	1808 (2.1)
[α-^{32}P]dGTP	1127 (1.0)	503	1229 (1.0)	1172 (1.0)
Band D_3				
[α-^{32}P]dTTP	550 (1.8)	300 (1.0)	647 (2.1)	662 (2.2)
[α-^{32}P]dCTP	387 (2.0)	140	755 (4.0)	538 (2.8)
[α-^{32}P]dATP	723 (4.3)	169 (1.0)	92	343 (2.0)
[α-^{32}P]dGTP	215 (1.0)	98	198 (1.0)	160 (0.8)

Depurination of D_2, D_3, or D_4 all resulted in one additional large, pyrimidine tract which was characterized as pT-C-C-C-Tp. When the label was in [^{32}P]TMP, the radioactivity in the tract was 50% phosphatase-sensitive (233 cpm in TpCpCpC$\overset{*}{p}$T and 196 cpm in $\overset{*}{P}_i$). Nearest neighbor analysis of this product gave greater than 80% of the radioactivity in dCp. When the products D_2, D_3, or D_4 were labeled with [^{32}P]CMP, the pyrimidine tract obtained was phosphatase-insensitive and the product gave a 2:1 ratio of counts in dCp and Tp (1153 cpm in dCp and 661 cpm in Tp). This same pyrimidine tract was isolated and characterized in the stepwise elongation experiments described above.

DISCUSSION

The approach involving nucleotide additions to a primer-template complex has been successfully used to determine the sequence of 23 nucleotide units beyond the C-C-A end of the gene for a tyrosine tRNA. The present work necessitated a slight revision in the previously determined sequence of the first 12 nucleotides. The mistake, unfortunately, was introduced during the two-step nucleotide addition experiments. The approach, using a restricted number of deoxyribonucleoside triphosphates, gave throughout error-free results when the experiments involved only one-step reactions. Thus, for example, the use of dTTP,

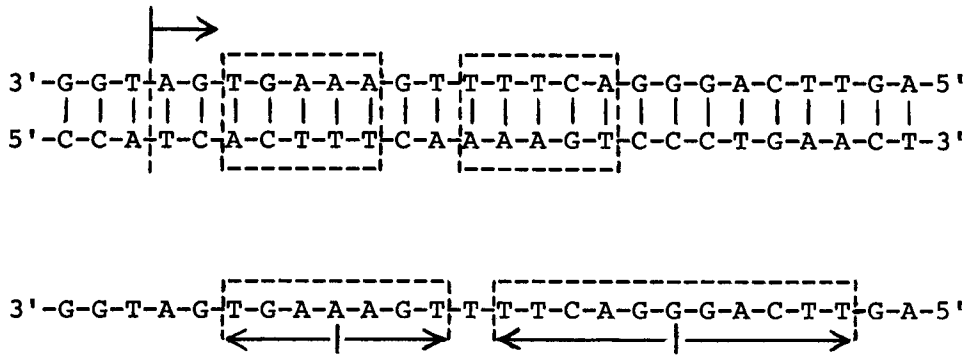

FIG. 11. Elements of symmetry in the nucleotide sequence beyond the 3′ end of the tyrosine tRNA gene. At the *top*, the double stranded configuration of this region is shown. The symmetric sections about an axis perpendicular to the page are indicated in the *dashed boxes*. The single strand region of the template r-strand is shown at the *bottom*. There are two symmetric regions indicated by the *dashed boxes*. Both regions possess two axes of symmetry at the center nucleotide, one of which is *perpendicular* to the page and the other in the *plane* of the page but *perpendicular* to the sequence.

dCTP, and dATP together gave a product which was shown to contain pyrimidine tracts, T-C and C-T-T-T-C, and 5 A units. However, to place the A units, it was necessary to carry out two-step incorporations. In this type of experiment, the product obtained from the first incorporation using one or two deoxyribonucleoside triphosphates had to be isolated free from the deoxyribonucleoside triphosphates before carrying out the second step. Possibilities for error could therefore arise at the second stage. Any contamination from the dNTPs, which were present at the first stage, would enhance incorporations at the second stage. Further, nicks could occur in the primer or the template DNA during the workup after the first stage so as to generate additional 3′-OH end groups. This would lead to new and unexpected incorporations. Thus, in the two-stage reactions, it becomes particularly important to purify the products of restricted nucleoside triphosphate addition, for example by polyacrylamide gel electrophoresis, before analysis. While this has been done in the present experiments, unfortunately, it was not done in the work reported previously.

Fortunately, the methods available for DNA sequencing are increasing rapidly and it is proving possible to verify the results obtained by one method with those obtained by an independent method. Thus, while basically only one approach was used in the work reported previously, in the present work, the sequencing of the first dodecanucleotide has been confirmed in at least two new ways. One method was the use of a primer (DNA-II) carrying a ribonucleotide unit at the 3′ end. Elongation of this primer followed by alkaline hydrolysis, purification of the product, and fingerprinting of its partial enzymic digests showed unequivocally the sequence and that of the A units in particular. A second method used was the incorporation of CMP in place of dCMP in chain elongation. As described, the series of oligonucleotides thus obtained provided further confirmation of the sequence. It should also be pointed out that repetition of the experiments described in the previous paper but including purification by gel electrophoresis also gave results all consistent with the revised sequence.

The sequence of the next 11 nucleotides, G-T-C-C-C-T-G-A-A-C-T, was derived by two methods. The first one again involved controlled nucleotide incorporation. Although the sequence could be deduced, the nearest neighbor analyses were not as clean as desired, partly because two-step reactions were invariably necessary. Elongation of the primer (DNA-I) with the four nucleoside triphosphates but using CTP in place of dCTP gave the sequences A-A-A-A-G-T-C and T-G-A-A-C, which provided confirmation of parts of the sequence derived above from the restricted dNTP incorporation. Further, the isolation of the pyrimidine tract, T-C-C-C-T, in two different experiments provided the necessary overlap to order the above oligonucleotides to a unique sequence.

The substitution of dCTP by CTP in DNA polymerase reaction is proving very useful in DNA sequencing (present work and Reference 7). It is particularly interesting that the chain elongation is slower in the presence of CTP than when dCTP is used and further, it seems to experience blocks along the template strand, perhaps where C units are to be incorporated. Thus, in the deoxyribopolynucleotides (R_2, R_3, and R_4) described above, the chains terminated just before C residues. Evidently, as soon as the C units in the sequence are inserted, the chain elongation proceeds at a high rate to the next site of ribonucleotide incorporation. The above circumstance is fortunate since it makes it possible, as demonstrated in the present work, to accumulate, relatively easily, discrete products of increasing chain length. However, as noted previously (8, 9) and again found in the present work, chain elongation in the presence of ribonucleoside triphosphates may sometimes come to a halt. Thus, in the presence of CTP, DNA-I could only be extended to a chain length of 44 to 45 nucleotide units. Neither increasing the CTP concentration to 150 μM CTP nor raising the temperature (20 and 37°) could overcome this block. This fact has so far placed a limit on the sequence determination in the present work using this approach.

Does the present sequence contain in it a part or all of the terminator sequence? While an answer can only come from transcription experiments *in vitro* using synthetically reconstructed systems, a few observations on the information available at this time may be made. Firstly, it may be noted that Altman (10, and unpublished work referred to in Reference 3) has isolated a precursor to the tyrosine tRNA from an RNase-minus mutant which is about 12 nucleotides longer at the C-C-A end. This discovery would imply that the termination signal is partly or completely included in the sequence herein described. Next, an examination of the present sequence leads to a number of interesting observations.

The region immediately following the C-C-A sequence is very A-T rich; thus, of the 13 base pairs, 10 are A-T base pairs. There then follows a G-C rich region, 5 G-C base pairs out of 7. The total sequence itself contains interesting elements of symmetry when viewed in its double-stranded and single-stranded forms. Thus, as seen in Fig. 11, the sequence as written in the double-stranded form has a region of symmetry (shown in *dashed boxes*) about a single axis. This type of symmetry has previously been found at the sites of action of a number of DNA restriction nucleases (11, 12) in the cohesive ends of λDNA (13) as well as the operator for the lactose operon.[2] If the present finding of

[2] W. Gilbert and co-workers, unpublished work.

the symmetrical bihelical region has a significance for the termination process, then it might be postulated that a termination factor such as ρ (14) would bind to this double-stranded region and perform its function by simply blocking the RNA polymerase. Previous evidence on this mode of action of the ρ factor (15, 16) is not conclusive, but the subject clearly needs further study.

The present sequence also possesses two regions which show symmetry when the sequence is viewed in the single-stranded form. These are also shown in Fig. 11 (*bottom*) for the top strand which would be the template strand for the RNA polymerase. These could be of significance in termination if the latter is induced by a specific nucleotide sequence on the strand being transcribed by the enzyme.

Finally, a third type of possibility for the mechanism is suggested by the experiments in which CTP was used along with the other three deoxyribonucleoside triphosphates. As described above, an effective block was encountered at the 44th to 45th nucleotide. Although the chain elongation in the presence of CTP is slow in general, the above block may be the consequence of a strong secondary structure in the DNA region ahead. The block experienced by the DNA polymerase may also affect the RNA polymerase.

All of the above observations can be tested by synthesizing suitable DNA duplexes and adding them to the C-C-A end of the DNA corresponding to the tyrosine tRNA.

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