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Studies on Polynucleotides

CXXII. THE DODECANUCLEOTIDE SEQUENCE ADJOINING THE C-C-A END OF THE TYROSINE TRANSFER RIBONUCLEIC ACID GENE*

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SUMMARY

Primer-template complexes were prepared by annealing synthetic deoxyribopolynucleotides with nucleotide sequences corresponding to the 3′ end of transfer ribonucleic acid^{tyr} to the r strand of ϕ80psu$_{III}$ DNA, which contains the tRNA^{tyr} gene (2). Using these systems, the nucleotide sequence beyond the 3′ end of the primers was determined by the DNA polymerase I-catalyzed addition of nucleotides by using a restricted number of deoxyribonucleoside 5′-triphosphates. In agreement with Altman and Smith (3), it was found that the sequence C-C-A is encoded in the tRNA^{tyr} gene. The sequence of the 12 nucleotides following the C-C-A end was shown to be T-C-A-A-C-T-T-T-C-A-A-A.

No information is available on the nucleotide sequences of the DNA regions which, presumably, signal the initiation and termination of transcription. If the nucleotide sequence of the DNA adjoining the above regions is known, for example, through transcription into messenger RNAs and transfer RNAs, one approach to the sequential analysis of the promoter and terminator regions is as follows. A deoxyribopolynucleotide segment with the above known DNA sequence and having suitable polarity may be synthesized. On hybridization with the appropriate separated strand of DNA, a template-primer relationship will be established, and the DNA polymerase can be used to extend the primer into the promoter or the terminator regions. The nucleotide sequences of the latter may then be deduced from the pattern of the nucleotide incorporation (2, 4).

The transducing phage, ϕ80psu$_{III^+}$ (5) carries the tyrosine suppressor tRNA gene, and the *in vivo* product of transcription of this gene has been identified as a precursor tRNA (6). The nucleotide sequence of the precursor has been determined (3) and the total synthesis of the DNA corresponding to it is in progress in this laboratory. Therefore, synthetic deoxyribopolynucleotide segments comprising the sequences at and near the 5′ and 3′ ends of the precursor tRNA are available for application of the above described approach to the determination of the nucleotide sequences in the promoter and terminator regions.

In a recent paper (2), initial work on the hybridization of several synthetic deoxyribopolynucleotides belonging to the 3′ end of the tRNA sequence with the separated r strand of the ϕ80psu$_{III}$ DNA was reported. The hybridization was shown to be specific to the expected site on the tRNA gene. In the present paper, the initial studies on the DNA polymerase I-catalyzed extension of the deoxyribopolynucleotide primers are reported. First, by using a primer which lacks the C-C-A sequence, it has been shown that this sequence is encoded in the tRNA gene. This finding is in agreement with the results of Altman and Smith (3), who found the above sequence in the precursor to the tyrosine tRNA. Second, the sequence of the 12 nucleotide units adjacent to the C-C-A terminus is shown to be T-C-A-A-C-T-T-T-C-A-A-A (Fig. 1).

EXPERIMENTAL PROCEDURE

Materials

Enzymes—DNA polymerase I from *Escherichia coli* was prepared according to the method of Jovin *et al.* (7) and was a gift from Dr. J. H. van de Sande and Dr. K. Kleppe. T_4 polynucleotide ligase and kinase were prepared from *E. coli* infected with bacteriophage T_4 am N82 according to the procedure of Richardson and co-workers (8, 9), and the kinase was a gift from Dr. J. H. van de Sande. Bacterial alkaline phosphatase, micrococcal nuclease, and spleen phosphodiesterase were obtained from Worthington Chemical Company. Snake venom phosphodiesterase was obtained from Calbiochem.

Nucleoside Triphosphates—[α-^{32}P]dTTP and ^{3}H-labeled dTTP and dCTP were obtained from New England Nuclear Corporation. [^{3}H]dATP was obtained from Schwartz Bioresearch, Inc. α-^{32}P-labeled dATP and dCTP were prepared by the procedure of Symons (10) as modified by Padmanabhan and Wu (11). [γ-^{32}P]ATP was prepared according to the published procedure (12). Unlabeled nucleoside triphosphates were obtained from P-L Biochemical Co. and Plenum Scientific Research, Inc., and where necessary were further purified as described by van de Sande *et al.* (13).

Oligonucleotides and Polynucleotides—The preparations of DNA II and of DNA III have been described previously. DNA I was

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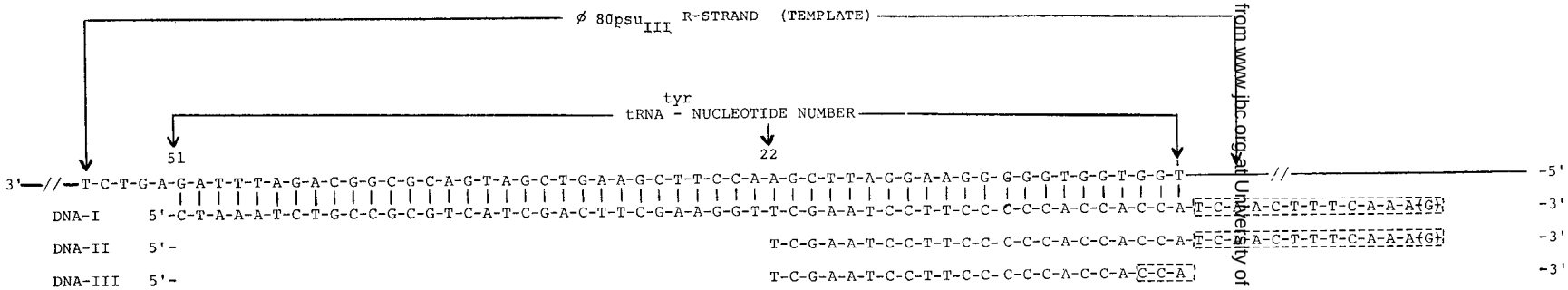

FIG. 1. Diagrammatic representation of the approach to the determination of nucleotide sequence at and beyond the 3′ terminus of the tyrosine tRNA gene. The ϕ80psu$_{III}$ r strand (*R strand*) is oriented in the 3′ to 5′ direction from *left* to *right*. Thus, the promoter regions (*not shown*) are to the *left*, and the terminator region is to the *right*. The nucleotide sequences of the three 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 three primers (*DNA-I* to *DNA-III*) are shown in the *dashed boxes*.

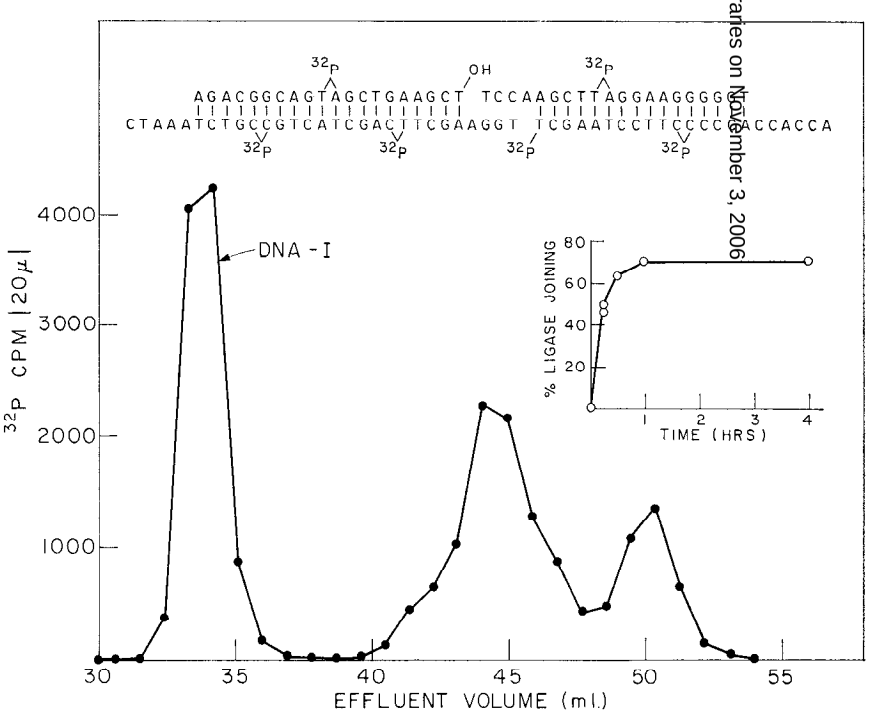

FIG. 2. Kinetics of synthesis and purification of DNA I (*DNA-I*). The reaction mixture contained 450 pmoles each of Duplex I and Duplex II (2), 50 mM Tris (pH 7.6), 0.033 mM ATP, 10 mM dithiothreitol, 10 mM $MgCl_2$, and 35 units of T_4 polynucleotide ligase in a total volume of 0.2 ml. The temperature was 5°. The kinetics were followed by DEAE-cellulose paper assay described previously (18). At the end of the reaction, EDTA (pH 8.0) was added to a concentration of 30 mM, and the mixture was loaded on top of a Sephadex-G75/SF column (100 × 1 cm). The column was pre-equilibrated with 10 mM potassium phosphate (pH 7.6) and was run at 65° with the same solvent at a flow rate of approximately 4 ml per hour. Void volume of the column was 29.0 ml. O——O, ^{32}P radioactivity.

prepared by ligase joining of Duplex II and Duplex IV containing segment 2 (2), with only Duplex IV being phosphorylated. In this way, one strand was longer than the other, facilitating its separation by column chromatography. The kinetics of joining and column separation are shown in Fig. 2. Degradation to 3′- and 5′-mononucleotides gave: dpA, 36 cpm; dpG, 48 cpm; dpT, 154 cpm; dpC, 1523 cpm; dGp, 31 cpm; dTp, 778 cpm; dCp, 2238 cpm; and dAp, 23 cpm. These polynucleotide sequences and their relation to the tRNA gene are shown in Fig. 1.

Methods

Nucleotide Incorporation Reactions—The polynucleotides (DNA I to III) were annealed to the r strand of ϕ80psu$_{III}$ in 80 mM NaCl-70 mM phosphate buffer (pH 6.9)-10 mM $MgCl_2$. The solution containing the DNA and the polynucleotide was first heated for 2 min at 100°, followed by incubation at 65° for DNA I or 60° for DNA II and DNA III. After 8 hours, the tubes were kept at 5° for 16 hours, followed by the addition of dithiothreitol to 10 mM, the various deoxyribonucleoside triphosphates to 1 μM, and DNA polymerase I to 30 units per ml. The concentration of ϕ80psu$_{III}$ r strand was 3 pmoles per ml. The same concentration of DNA I was used, whereas a 3-fold excess of DNA II and DNA III was used. The total volume of the reaction mixtures was usually 100 or 200 μl, and the temperature was 5°.

Kinetics of Nucleotide Incorporations—The kinetics of nucleotide incorporations were followed by pipetting 10- or 20-μl aliquots containing 0.03 to 0.06 pmoles of DNA onto DE 81 paper strips (Whatman) previously spotted with 50 μl of a solution of 50 mM EDTA-50 mM sodium pyrophosphate. The strips were then irrigated with 0.5 M ammonium formate-7 M urea-1 mM EDTA-10 mM sodium pyrophosphate for 2 to 3 hours. The origins of the strips were cut out and counted in a liquid scintillation counter. If the product was ^{3}H-labeled, the paper was oxidized in a Packard model 305 Tri-Carb Sample Oxidizer and counted.

Isolation of Elongated DNAs—For further characterization, the products from the polymerase reactions were separated from the excess nucleoside triphosphates on a column (5 ml pipette) of Sephadex G-50/F (Pharmacia). The columns were pre-equilibrated with 0.1 M triethylammonium bicarbonate and eluted with the same solution at 25°. Before loading on the column, the reaction mixtures were made 50 mM in EDTA and then heated to 100° for 2 min and cooled to room temperature.

Nearest Neighbor Frequency Analysis—The nearest neighbor analyses were performed by using the procedure described by Kleppe *et al.* (14), except that triethylammonium bicarbonate (pH 9.5) was used in place of sodium glycinate. The 3′-nucleotides and nucleosides were separated by two dimensional paper chromatography as described by Wu (15).

Diphenylamine-Formic Acid Degradation—The procedure was essentially as described by Burton (16) and used by Ling (17). The products from this reaction were separated by electrophoresis on DE 81 paper in 7% formic acid. The paper strips were scanned by a Packard model 7200 Radiochromatogram Scanner, and the radioactive spots were cut out and eluted with 2 M triethylammonium bicarbonate (pH 7.6) for further analysis.

RESULTS

Primer-dependent Polymerase-catalyzed Incorporations of Nucleotides—It was important to establish at the outset that the nucleotide incorporations were primer-dependent. The labeled nucleotide chosen for study was [^{3}H]dTTP because the first nucleotide to the right of C-C-A in the tyrosine tRNA precursor has been found to be U (3). As seen in Fig. 3, in the presence of this labeled nucleotide and the primer (DNA-I) rapid incorporation of approximately 1 mole of dTMP occurred while the incorporation in the absence of the primer was negligible. The background incorporation increased somewhat when the three dNTPs, [^{3}H]-dTTP + dATP + dCTP, were provided and there was a further increase when all the four dNTPs were present. However, the incorporations observed were, in both cases, only about 10% or less of those obtained in the parallel experiments in which the primers were present (see Fig. 8).[1]

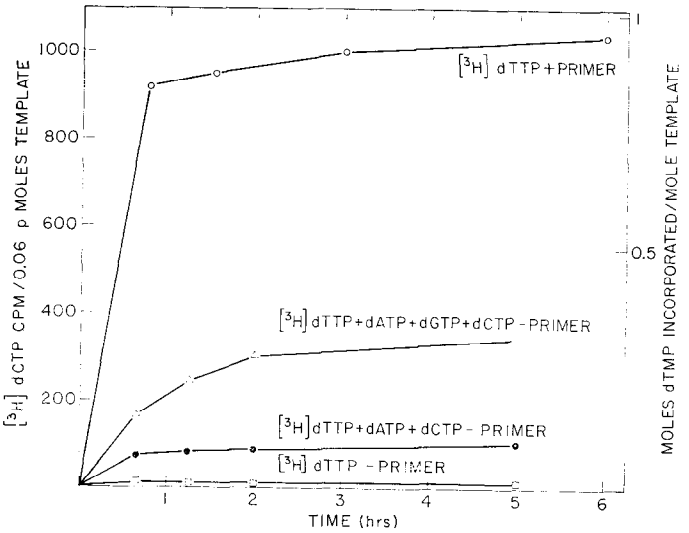

FIG. 3. Nucleotide incorporation in the presence and absence of the primer (DNA I) and ϕ80psu$_{III}$ r strand [^{3}H]dTTP was used as the labeled nucleotide, and it was used alone when DNA I was hybridized to the r strand (○——○). Incorporation of [^{3}H]dTTP, with no primer present (□——□), in the presence of dATP and dCTP (●——●), and in the presence of dATP, dCTP, and dGTP (△——△) are also shown.

Further, it was necessary to show that the nucleotide incorporations represented elongation of the primer. This has been done in the experiments described later by (*a*) physical separation of the radioactively labeled extended primer and (*b*) its characterization in regard to its size and nucleotide sequence (Figs. 11 and 12).

3′-Terminal Sequence Encoded in the tRNA Gene—The 3′-terminal sequence, C-C-A, is encoded in the tRNA gene. A direct proof that the terminal C-C-A sequence is coded for by the tRNA gene was obtained by using DNA III, a 19-nucleotide-unit-long polynucleotide which lacks the C-C-A sequence, as the primer, and ϕ80psu$_{III}$ r strand, as the template. DNA polymerase-I catalyzed the addition of the above sequence to the 3′ end of DNA I.

Fig. 4 shows the kinetics of incorporation of [^{3}H]dCTP alone and of [^{3}H]dCTP in the presence of [^{3}H]dATP. The two deoxyribonucleoside triphosphates used had equal specific activity. It can be seen that about 1.8 moles of dCMP were incorporated per mole of the template when only [^{3}H]dCTP was present. When

[1] In some experiments using the l strand of ϕ80psu$_{III}$ DNA (R. C. Miller and J. H. van de Sande, unpublished observation), considerable incorporations in the absence of the primers were observed, especially when the l strand had been annealed in 2 × 0.1 M NaCl + 0.015 M sodium citrate at 65° for 16 hours followed by dialysis into the repair replication buffer. Even with the r strand, in some older preparations which have been stored for up to 1 year, increased incorporation may be obtained. Frequent checks of nucleotide incorporations in the absence of the primer are desirable.

both [³H]dCTP and [³H]dATP were present, about 3 moles of nucleotide were incorporated. The degradation of the product after the nucleotide incorporation to the 3′-mononucleotides gave the results shown in Table I. In Experiment 1, in which only [³H]dCTP was supplied to the reaction mixture, a dCp to dC ratio of 1.13 was found for the radioactivity. This result shows that 2 dCMP units were incorporated to give the sequence pCpC. In Experiment 2, in which both dCTP and dATP were added, degradation showed bulk (80%) of the radioactivity to be present in dCp and dA, the ratio of radioactivity in dCp to dA being 2.23. Some radioactivity was unexpectedly found in dC and dAp. The radioactivity in dC could be due to the incomplete "covering" of the C-C sequence by the terminal A unit, whereas the radioactivity in dAp could be due to the significant exchange of the terminal A unit in DNA III with radioactive dAMP before the addition of C-C sequence.

In further experiments, [α-³²P]dCTP alone or [α-³²P]dATP and dCTP were used. Experiment 3 in Table I shows the results obtained on nearest neighbor analysis of the product obtained when [α-³²P]dCTP alone was used. As expected, the radioactivity was in dAp and dCp, the ratio found being 1.3. The result may again indicate that the incorporation of the terminal nucleotide does not go to completion. In Experiment 4, in which [α-³²P]-dATP and dCTP were used, the bulk (85%) of the counts were in dCp, although some radioactivity was found in dAp.

The above results are all consistent with the terminal C-C-A sequence being encoded in the tRNA gene.

First Nucleotide after C-C-A—The first nucleotide after C-C-A is T. In this and most of the experiments, DNA I was used as the primer, the template being $\phi 80psu_{III}$ r strand.

The kinetics of nucleotide incorporation when [³H]dTTP was used alone, or in the presence of one more dNTP (dCTP or dATP) or two more dNTPs (dCTP + dATP), are shown in Fig. 5. The extent of incorporation of [³H]dTTP was the same (about 1 mole of the primer) when this dNTP was used alone or in the presence of dCTP or dATP. However, the incorporation was increased to about 4 moles when both dATP and dCTP were added. The products, when degraded to 3′-nucleotides and nucleosides, gave the results shown in Table II. More than 90%

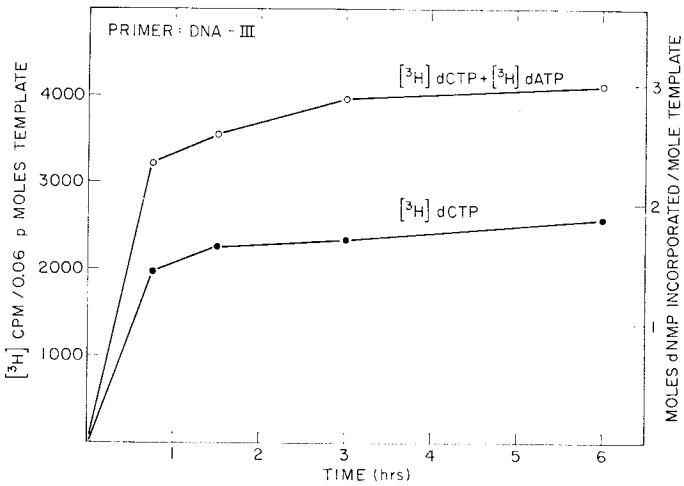

FIG. 4. Kinetics of nucleotide incorporation into DNA III (*DNA-III*) using DNA polymerase I. The conditions are described under "Methods." [³H]dCTP was used alone (●——●) and with [³H]dATP (○——○).

TABLE I

3′-Nucleotide and nucleoside analyses of products obtained by elongation of DNA-III using [³H]- or [α-³²P]dCTP and [³H]- or [α-³²P]dATP as labeled nucleotides

Experiment No.	dNTPs used	Radioactivity			
		dAp	dCp	dA	dC
		cpm			
1	[³H]dCTP		4,555 (1.13)[a]		4,048 (1.00)
2	[³H]dCTP + [³H]dATP	1,062	6,036 (2.23)	2,614 (1.00)	1,161
3	[α-³²P]dCTP	41,971 (1.29)	32,275 (1.00)		
4	[α-³²P]dATP + dCTP	2,318	14,457		

[a] The numbers in parentheses are the observed molar ratios. The conditions for nucleotide incorporations, isolation of the products, and nearest neighbor analysis are given under "Methods." The kinetics of incorporations are shown in Fig. 4.

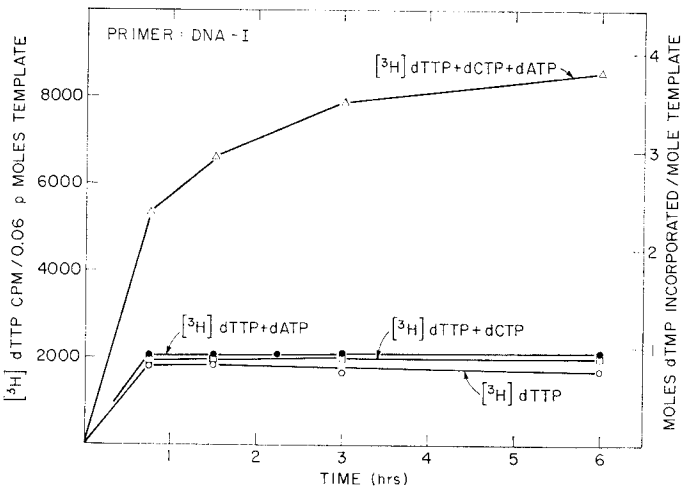

FIG. 5. Kinetics of the repair of DNA I (*DNA-I*) using *Escherichia coli* DNA polymerase I and [³H]dTTP as the labeled nucleotide. The conditions are described under "Methods." The incorporation of [³H]dTTP was studied alone (○——○), in the presence of dCTP (□——□), in the presence of dATP (●——●), and in the presence of dCTP and dATP (▲——▲).

TABLE II

3′-Nucleotide and nucleoside analyses of products obtained by elongation of DNA I using [³H]- or [α-³²P]dTTP as labeled nucleotide

The conditions for incorporation, isolation of the products and nearest neighbor analyses are given under "Methods." The kinetics of nucleotide incorporations are shown in Fig. 5.

Experiment No.	dNTPs used	Radioactivity				
		dAp	dGp	dTp	dCp	dT
		cpm				
1	[³H]dTTP			167		2681
2	[³H]dTTP + dATP			191		2899
3	[³H]dTTP + dCTP			2255		237
4	[³H]dTTP + dCTP + dATP			6862		437
5	[α-³²P]dTTP	7055	237	318	263	

of the radioactivity was released as thymidine in Experiments 1 and 2. However, when dCTP was present together with [^{3}H]-dTTP, the radioactivity was now found in dTp (>90%). This finding together with the fact that only one mole of [^{3}H]dTMP is incorporated in the presence of dCTP (Fig. 5) shows that at least 1 dCMP unit is incorporated adjacent to the initial dTMP residue.

In the next experiment in which [^{3}H]dTTP was used together

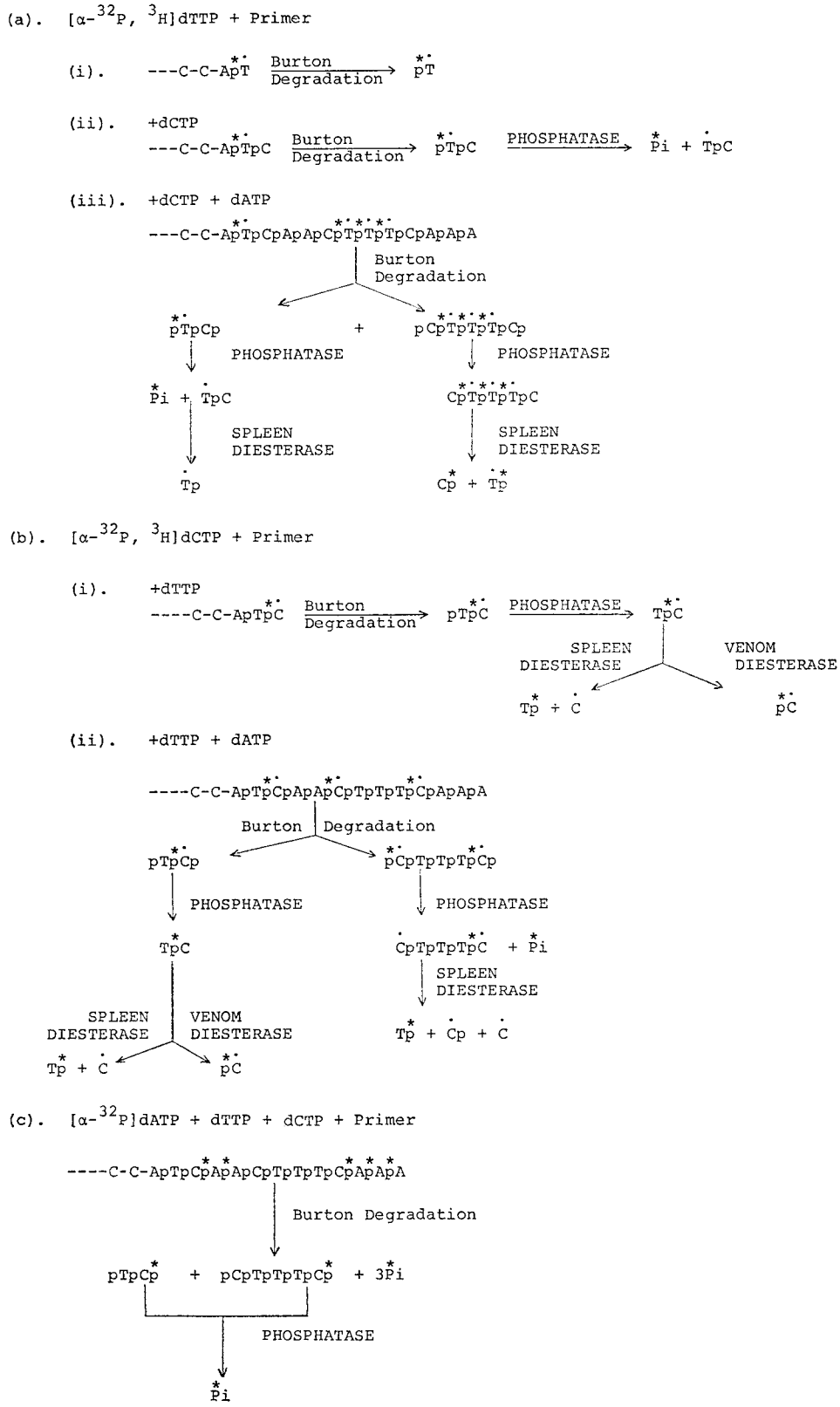

FIG. 6. Description of the origin of degradation products of the various labeled repair products by the Burton depurination procedure. The conditions are described under "Methods." The abbreviation - - - C-C-A denotes the whole primer, DNA I (or DNA II), and the following nucleotides are those inserted by *Escherichia coli* DNA polymerase I using the ϕ80psu$_{III}$ r strand as template. The symbols $\mathring{p}\dot{N}$ stand for [^{32}P, ^{3}H] N, where N is dA, dC, or dT.

with dATP + dCTP (Experiment 4, Table II), most of the radioactivity was again found in dTp. Since 4 moles of [^{3}H]dTMP are incorporated (Fig. 5), the results show that a longer chain is synthesized in the presence of the three dNTPs.

An experiment was also performed using [α-^{32}P]dTTP, and the results of nearest neighbor analysis are shown in Table II (Experiment 5). Radioactivity was found mostly in dAp, as expected. Burton degradation (pyrimidine nucleotides and clusters) gave the radioactivity in dpT (Fig. 6). The results are all consistent with T being the first nucleotide following C-C-A.

Sequence of Two Nucleotides after C-C-A—The sequence of the two nucleotides after C-C-A is T-C. The results in the preceding section indicated that the nucleotide T was followed by 1 or more C units. Fig. 7 shows the kinetics of incorporation of [^{3}H]dCTP alone, or in the presence of dTTP or in the presence of dTTP and dATP. In agreement with the results described above, no incorporation of dCTP occurs unless dTTP is present. The presence of both dATP and dTTP leads to an increased incorporation of [^{3}H]dCTP. The product obtained by using [^{3}H]dCTP and dTTP was degraded to 3′-nucleotides and nucleosides, and the results obtained are shown in Table III (Experiment 1). The radioactivity was mostly in dC. This result shows (*a*) that the addition of the first T nucleotide to the C-C-A end is followed by the addition of a single C nucleotide, and (*b*) that C is followed neither by C nor by T. The 3′ analysis of the product obtained by using [^{3}H]dCTP, dTTP, and dATP is also shown in Table III (Experiment 2). Most of the radioactivity was now in dCp. This result indicates that there is an A unit after the TpC sequence. Furthermore, the increased incorporation (about 3 moles) of [^{3}H]dCTP in the presence of dATP and dTTP shows that additional dCMP units are incorporated into the longer sequence now synthesized.

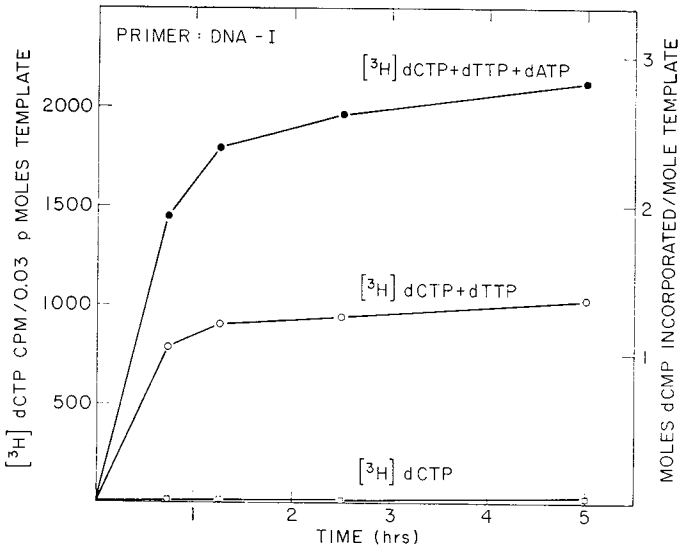

FIG. 7. Kinetics of the repair of DNA I (*DNA-I*) using [^{3}H]dCTP as the labeled nucleotide alone (□——□), in the presence of dTTP (○——○), and in the presence of dTTP and dATP (●——●). The conditions are described under "Methods."

TABLE III

3′-Nucleotide and nucleoside analyses of products obtained by elongation of DNA I using [^{3}H]- or [α-^{32}P]dCTP as labeled nucleotide

The conditions for the synthesis, isolation of the products, and nearest neighbor analyses are given under "Methods." The kinetics of incorporation are shown in Fig. 7.

Experiment No.	dNTPs used	Radioactivity				
		dAp	dGp	dTp	dCp	dC
		cpm				
1	[^{3}H]dCTP + dTTP				547	3398
2	[^{3}H]dCTP + dTTP + dATP				6258	943
3	[α-^{32}P]dCTP + dTTP	449	56	5517	165	

When [α-^{32}P]dCTP was incorporated in the presence of dTTP alone (kinetics not shown) and the product was degraded to 3′-mononucleotides, the majority of the counts (90%) were found in dTp (Table III, Experiment 3). Burton degradation of this product gave a dinucleotide which was characterized as dpT$\overset{*}{p}$C[2] (Fig. 6). Thus, it had a mobility on electrophoresis identical with that of a chemically synthesized marker. Treatment with bacterial alkaline phosphatase gave d$\overset{*}{p}$C which, again, co-electrophoresed with an authentic sample. Further, spleen phosphodiesterase treatment of dT$\overset{*}{p}$C gave dT$\overset{*}{p}$ as the only radioactive product. Venom phosphodiesterase gave d$\overset{*}{p}$C as the only radioactive product.

Sequence Next to T-C—The sequence next to T-C is A-A. The sequence is C-C-A-T-C-A-A. The results described above on nucleotide incorporations when three dNTPs were present indicated that an A unit followed the T-C sequence. To demonstrate A incorporation and yet to restrict the chain elongation which evidently occurred in the presence of the three nucleoside triphosphates, the following experiment was carried out. By using DNA I as primer, the nucleotide incorporation was carried out using unlabeled dTTP and dCTP. The product, ϕ80psu$_{III}$ r strand hybrid was separated from the excess of nucleoside triphosphates by gel filtration and now subjected to a DNA polymerase, catalyzed reaction using [α-^{32}P]dATP (Table IV). Nearest neighbor analysis of the product gave the radioactivity in dCp and dAp, and the ratio was 1.05. Further, Burton degradation of the radioactive product gave pTpC$\overset{*}{p}$ (7970 cpm) and P$_i$ (5386 cpm) in addition to about 20% of the radioactivity in other smaller products. The product, pTpC$\overset{*}{p}$, had the expected electrophoretic mobility and, on treatment with the phosphatase, released all of the radioactivity as $\overset{*}{P}_i$. The results are all consistent with a sequence of A-A following T-C.

Nucleotide Unit Adjacent to T-C-A-A—The nucleotide unit adjacent to T-C-A-A is C, hence the sequence is C-C-A-T-C-A-A-C. The above method involving (*a*) nucleotide incorporation using unlabeled dNTPs, (*b*) isolation, and (*c*) repeat incorporation with labeled dNTPs was again used to obtain information on the nucleotide adjoining A-A sequence. DNA I was first extended using unlabeled dTTP and dCTP. The product was isolated free from the excess dNTPs and incorporation was again performed by using unlabeled dATP and [α-^{32}P]dCTP.

Nearest neighbor analysis of the product showed that the majority of the counts (70%) were in dAp. (Table IV, Experiment 1b). The remaining counts were spread in dGp, dTp, and dCp. Burton degradation of the product gave the bulk of the counts in d$\overset{*}{p}$C, although there were other minor radioactive products.

Dodecanucleotide Sequence Next to C-C-A: T-C-A-A-C-T-T-T-C-A-A-A

Limited Primer Elongation Using Three Deoxyribonucleoside Triphosphates, dATP, dCTP, and dTTP—Two experiments on

[2] The symbol $\overset{*}{p}$N denotes [^{32}P]pN, where N is dA, dC, or dT.

TABLE IV

3′-Nucleotide and nucleoside analyses of products obtained by technique of stepwise nucleotide additions to DNA I

Experiment No.	dNTPs used	Radioactivity				
		dAp	dGp	dTp	dCp	dA
		cpm				
1						
Step 1	dTTP + dCTP					
Step 2	(*a*) [α-^{32}P]dATP	5967 (1.05)[a]	1579	1989	5651 (1.00)	
	(*b*) [α-^{32}P]dCTP + dATP	9894	778	3372	1764	
2						
Step 1	dTTP + dCTP					
Step 2	dCTP + dATP					
Step 3	[^{3}H, α-^{32}P]dATP + dTTP + dCTP	[^{32}P] 4459 (1.75)	1160	819	2552 (1.00)	
		[^{3}H] 2763 (2.05)				1352 (1.00)

[a] The numbers in parentheses are the observed molar ratios. The conditions for synthesis, isolation of the products, and nearest neighbor analysis are given under "Methods."

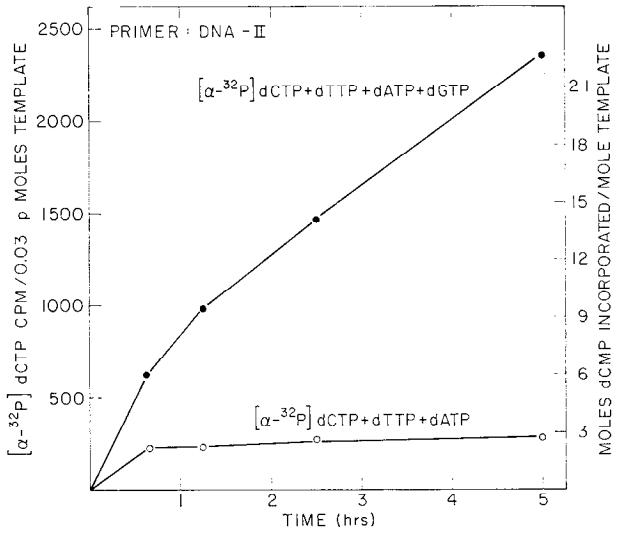

FIG. 8. Kinetics of the repair of DNA I using [α-^{32}P]dCTP as the labeled nucleotide in the presence of dTTP and dATP (○——○) and in the presence of dTTP, dATP, and dGTP (●——●). The conditions are described under "Methods."

nucleotide incorporation using three deoxyribonucleoside triphosphates have already been shown, one in which [^{3}H]dTTP was the radioactively labeled dNTP (Fig. 5) and the second in which [^{3}H]dCTP was the labeled dNTP (Fig. 7). In both experiments, the synthesis leveled off with only a few moles of the labeled nucleotides being incorporated. In Fig. 8, two further experiments are shown, in which the incorporation obtained using the above mentioned three dNTPs is compared with that obtained using all four dNTPs. [α-^{32}P]dCTP was used as the labeled nucleotide. As is seen with dCTP, dATP, and dTTP, the reaction reached a decisive plateau when 3 moles of dCMP had been incorporated, and this result confirmed that in Fig. 7. However, when the fourth nucleotide, dGTP, was also present, the synthesis was much more extensive and no plateau was discernible even when more than 20 moles of dCMP had been incorporated.

In the following, the sequence of the oligonucleotide block added to the primer (DNA-I and DNA-III) when dATP, dCTP and dTTP are provided is shown to be T-C-A-A-C-T-T-T-C-A-A-A.

Characterization of Pyrimidine Tracts in Product Synthesized Using [α-^{32}P, ^{3}H]dTTP, dATP, and dCTP—Burton degradation of the product obtained using DNA I as the primer and [^{3}H, α-^{32}P]dTTP, dATP, and dCTP gave two pyrimidine blocks, which were separated by electrophoresis on DE-81 paper in 7% formic acid and identified as $\overset{*}{p}\dot{T}pCp$ and $pC\overset{*}{p}\dot{T}\overset{*}{p}T\overset{*}{p}\dot{T}pCp$[3] (Fig. 6). Thus, the two products contained radioactivity (both ^{3}H and ^{32}P) in the ratio 1:2.6 (539 cpm of ^{3}H, 6575 cpm of ^{32}P in $\overset{*}{p}\dot{T}pCp$; 1409 cpm of ^{3}H, 16196 cpm of ^{32}P in $pC\overset{*}{p}\dot{T}\overset{*}{p}T\overset{*}{p}\dot{T}pCp$). The ^{32}P radioactivity in the smaller product was completely sensitive to the phosphomonoesterase, and the resulting product, $\dot{T}pC$, on degradation with the spleen phosphodiesterase gave $d\dot{T}p$ as the only radioactive product. The larger product did not contain any ^{32}P in the monoesterified form and after phosphatase treatment gave $C\overset{*}{p}\dot{T}\overset{*}{p}T\overset{*}{p}\dot{T}pC$. The latter on treatment with the spleen phosphodiesterase gave $d\dot{T}\overset{*}{p}$ and $dC\overset{*}{p}$ in the ratio 1.92 (5350 cpm: 2787 cpm), and all of the ^{3}H radioactivity was in $d\dot{T}p$, with no $d\dot{T}$ being formed (Fig. 6) (401 cpm in $d\dot{T}p$ and 4 cpm in $d\dot{T}$).

Characterization of Pyrimidine Tracts in Product Synthesized Using [α-^{32}P, ^{3}H] dCTP, dTTP, and dATP—Burton degradation of the product thus obtained gave the same two pyrimidine blocks as identified above. Thus, the two products (Fig. 6) contained radioactivity in a nearly 1:2 ratio (734 cpm of ^{3}H, 21,460 cpm of ^{32}P in $pT\overset{*}{p}\dot{C}p$; 1,452 cpm of ^{3}H, 33,448 cpm of ^{32}P in $\overset{*}{p}\dot{C}pTpTpT\overset{*}{p}\dot{C}p$). The ^{32}P in $pT\overset{*}{p}\dot{C}p$ was completely insensitive to the phosphatase. The product ($T\overset{*}{p}\dot{C}$) thus obtained had electrophoretic mobility identical to that of an authentic synthetic sample. On degradation with the spleen and venom phosphodiesterases, the radioactive mononucleosides and nucleosides formed were exactly as expected (Fig. 6).

The larger product had 50% of its ^{32}P radioactivity phosphatase sensitive (643 cpm as $CpTpTpT\overset{*}{p}C$ and 645 cpm as $\overset{*}{P}_i$), and the resulting product migrated faster on DE-81 paper. The electrophoretic mobility after phosphatase treatment was consistent with that of $CpTpTpT\overset{*}{p}C$ at pH 1.9 (Fig. 6). Partial

[3] The symbol $p\dot{N}$ denotes [^{3}H]pN where N is dA, dC, or dT.

spleen phosphodiesterase degradation gave a mixture of products, whereas complete digestion gave only dT$\overset{*}{p}$ and a ratio dCp to dC of 1 to 1 (581 cpm as 579 cpm).

Characterization of Product Synthesized Using [α-^{32}P]dATP, dTTP, and dCTP—The sequence T-C-A-A-C following C-C-A has been deduced above. Since only two pyrimidine blocks are found in the product synthesized using three deoxyribonucleoside triphosphates and of these only the pentanucleotide (Fig. 6 and see above) contains C at its 5′ end, this block must be directly linked to the A-A sequence above. The total sequence, which can be concluded from the evidence given so far is T-C-A-A-C-T-T-T-C. Information on further sequence was obtained using radioactively labeled [^{3}H]dATP or [α-^{32}P]dATP.

When the product obtained by using [^{3}H] or [α-^{32}P]dATP, dCTP, and dTTP was degraded to 3′-nucleotides and nucleosides, radioactivity was found in dAp and dA in a ratio of 3.52 (Table V). This result indicated that dA was present at the 3′-hydroxyl end and that there were probably a total of 5 dA units in the chain.

Burton degradation of the product obtained using [α-^{32}P]dATP, dTTP and dCTP gave three products: these were pTpC$\overset{*}{p}$, pCpTpTpTpC$\overset{*}{p}$ (Fig. 6), and $\overset{*}{P}_i$. The ratio of the radioactivity was 1.0 to 1.1 to 2.9 (6464, 7163, and 18918 cpm). This result indicated that the 3 A units present at the end of the chain were adjacent to one another.

A further check of the above sequence (three contiguous A units following T-C-A-A-C-T-T-T-C) was carried out in the following three step experiment: (*a*) primer elongation with dTTP and dCTP followed by isolation and reannealing of the template and the primer; (*b*) incorporation with dCTP and dATP followed by isolation and reannealing, and (*c*) a final reaction with dTTP, dCTP, and [^{3}H, α-^{32}P]dATP. The nearest neighbor analysis of the product gave a ratio of dA$\overset{*}{p}$ to dC$\overset{*}{p}$ of 1.75 to 1 and a ratio of dA̋p to dA̋ of 2.05 to 1 (Table IV, Experiment 2).

Nearest Neighbor Analyses of ^{32}P-Labeled Products

Nearest neighbor analyses of ^{32}P-labeled products confirm the sequence C-C-A-T-C-A-A-C-T-T-T-C-A-A-A. Use has been made above of the products obtained by using one of the three deoxyribonucleoside triphosphates (dTTP, dCTP, dATP) labeled with ^{32}P in the α-phosphate and the other two unlabeled. Further data obtained with these products are shown in Fig. 9 and Table V. By using [α-^{32}P]dTTP in the presence of dCTP and dATP, the incorporation of 4 moles of dTMP per mole of the template was observed (Fig. 9). Nearest neighbor analysis (Table V, Experiment 1) gave radioactivity in dAp, dTp, and dCp with the ratio 1.21:1.92:1. Synthesis with [α-^{32}P]dCTP in the presence of dTTP and dATP resulted in the incorporation of 3 moles of dCMP (Fig. 9), and the nearest neighbor analysis of the product gave radioactive dTp and dAp in the ratio 2.38 (Table V).

Labeling with [α-^{32}P]dATP in the presence of dCTP and dTTP resulted in about 4.6 moles of dAMP per mole of the template being incorporated (Fig. 9). The nearest neighbor analysis showed radioactivity (Table V, Experiment 3) in dAp and dCp with the ratio of 2.60 to 2.00 (Table V, Experiment 3).

TABLE V

3′-Nucleotide and nucleoside analyses of products obtained by elongation of DNA I using dTTP, dCTP, and dATP

Experiment No.	dNTPs used	Radioactivity				
		dAp	dGp	dTp	dCp	dA
		cpm				
1	[α-^{32}P]dTTP + dATP + dCTP	5863 (1.21)[a]	136	9581 (1.92)	4959 (1.00)	
2	[α-^{32}P]dCTP + dATP + dTTP	1912 (1.00)	41	4530 (2.38)	206	
3	[α-^{32}P]dATP + dCTP + dTTP	6324 (2.60)	830	840	4698 (2.00)	
4	[^{3}H]dATP + dCTP + dTTP	2950 (3.52)				842 (1.00)

[a] The numbers in parentheses are the observed molar ratios. The conditions for synthesis, isolation of the products, and nearest neighbor analyses are given under "Methods." The kinetics of repair are shown in Fig. 9.

Nearest Neighbor Analyses of ^{32}P-Labeled Products Using DNA II as Primer

In all of the work described so far, DNA I had been used as the primer. The shorter primer, DNA II, (22 nucleotide units long) was used in the present experiment. The kinetics and total incorporation of each one of the three α-^{32}P-labeled nucleoside triphosphates in the presence of the other two dNTPs are shown in Fig. 10. The results are practically identical to those described above with DNA I in Fig. 9.

The nearest neighbor analyses of the various products as well as the results of Burton degradation are given in Table VI. Again, these are very nearly the same as found above for the products obtained using DNA I.

Isolation and Characterization of Extended Primers

In final characterization of the products formed, their physical isolation and size characterization was carried out. DNA II was elongated by using [α-^{32}P]dCTP, dTTP, and dATP; the product

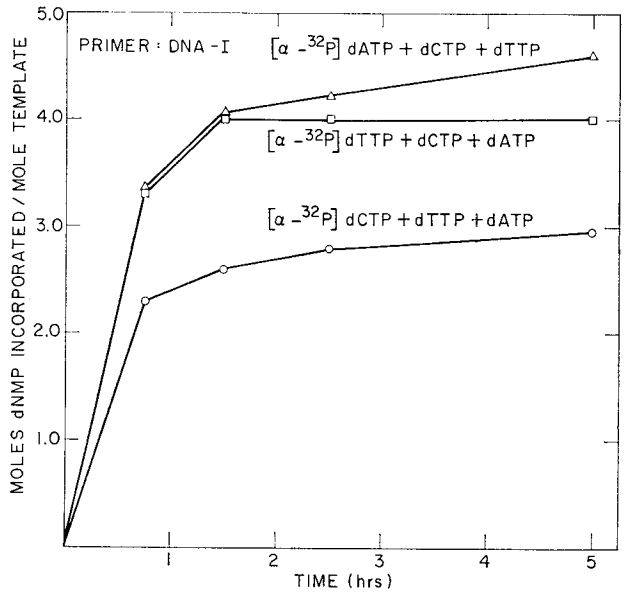

FIG. 9. Kinetics of the repair of DNA I (*DNA-I*) with α-^{32}P-labeled deoxyribonucleoside triphosphates. [α-^{32}P]dCTP was used in the presence of dTTP and dATP (○——○); [α-^{32}P]dTTP was used in the presence of dCTP and dATP (□——□); [α-^{32}P]-dATP was used in the presence of dCTP and dTTP (△——△). The conditions are described in "Methods."

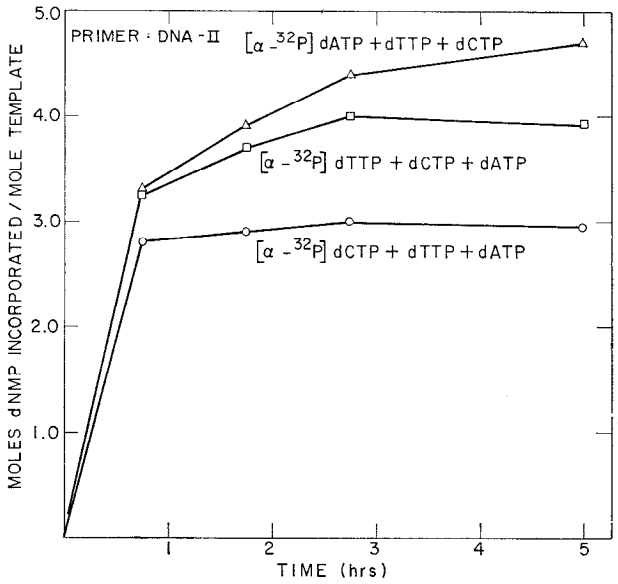

FIG. 10. Kinetics of the repair of DNA II (*DNA-II*) with α-^{32}P-labeled deoxyribonucleoside triphosphates. [α-^{32}P]dCTP was used in the presence of dTTP and dATP (○——○); [α-^{32}P]dTTP was used in the presence of dCTP and dATP (□——□); [α-^{32}P]-dATP was used in the presence of dCTP and dTTP (△——△). The conditions are described under "Methods."

was heat-denatured, chilled, and chromatographed on a 10-ml agarose 1.5m column with calf thymus DNA and tRNA as markers. The elution pattern is shown in Fig. 11. There is a small amount of radioactivity in the region of DNA, but the majority of the counts (93%) is smaller than tRNA. This result and the one shown below using 5′-^{32}P-labeled primers confirm that this nucleotide incorporation represents addition (elongation) of the primer and that only about 10% or less of the incorporated radioactivity goes into the DNA.

Extension of the DNA I using the three dNTPs also gave a product which could be separated from the r strand using electrophoresis on cellulose acetate. Again the pattern of radioactivity showed that most (>90%) of the radioactivity was present in the extended primer.

Extended primers isolated as above (gel filtration and electrophoresis) were subjected to nearest neighbor analysis (Table VII). The results were again as expected for the dodecanucleotide sequence. Burton degradation also gave the expected distribution of radioactivity.

The radioactive product from the extension of DNA I labeled with [α-^{32}P]dCTP and [α-^{32}P]dATP were subjected to electrophoresis by Dr. T. Maniatis on polyacrylamide gels in 7 M urea. The product from DNA I (expected size, 63 nucleotides) migrated midway between two synthetic polynucleotides of size 60 and 66 nucleotides (Fig. 12) (20). The product from DNA II labeled with [α-^{32}P]dCTP migrated a distance expected for a polynucleotide 33 to 35 nucleotides in length.

In the experiments in which DNA II was used as the primer, a 2-fold excess of DNA II over the r strand had been used. In order to confirm that the primer was utilized by the DNA polymerase for chain elongation only when the latter was bound to the r strand at the tRNA site, the following experiment was performed. DNA II was labeled at the 5′ end with ^{32}P-phosphate group using polynucleotide kinase. This was now extended in a standard polymerase reaction using unlabeled dTTP, dCTP, and dATP. The isolated [^{32}P]polynucleotides were then subjected to electrophoresis on 15% polyacrylamide gels in 7 M

TABLE VI

Nearest neighbor analyses and Burton degradation analyses of products obtained by elongation of DNA II using dTTP, dCTP, and dATP

The numbers in parentheses are the observed molar or product ratios. The conditions for nucleotide incorporation isolation, nearest neighbor analysis, and depurination are given in "Methods." The kinetics of incorporations is shown in Fig. 9.

a. Nearest neighbor analyses

Experiment	dNTPs used	Radioactivity			
		dAp	dGp	dTp	dCp
		cpm			
1	[α-^{32}P]dCTP + dTTP + dATP	25,476 (1.00)	1,191	55,877 (2.18)	5,114
2	[α-^{32}P]dTTP + dCTP + dATP	10,982 (1.32)	406	14,960 (1.81)	8,234 (1.00)
3	[α-^{32}P]dATP + dCTP + dTTP	33,385 (3.09)	3,821	3,552	24,738 (2.00)

b. Burton degradation products

Experiment	dNTPs added	Radioactivity		
		pCpTpTp-TpCp	pTpCp	Miscellaneous
		cpm		
1	[α-^{32}P]dCTP + dTTP + dATP	123,147 (1.82)	66,874 (1.00)	31,613
2	[α-^{32}P]dTTP + dCTP + dATP	26,891 (2.84)	9,468 (1.00)	2,707

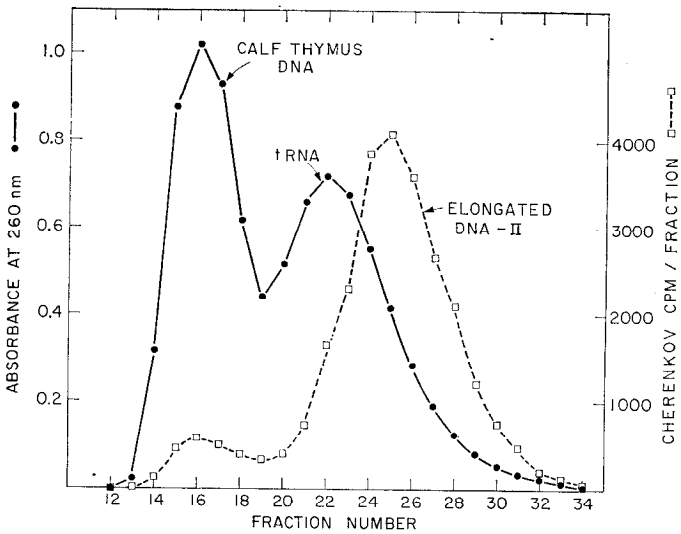

FIG. 11. Column chromatographic separation of the products formed by using DNA II (*DNA-II*) as the primer. The reaction mixture was separated from the excess deoxyribonucleoside triphosphates on a G50/F column ("Methods"). This sample was then mixed with calf thymus DNA and crude *Escherichia coli* tRNA, boiled for 5 min, followed by chilling and column chromatography. The column was 1.5 M agarose in a 10-ml calibrated pipette, pre-equilibrated with 0.025 M triethylammonium bicarbonate (pH 7.6), and was run at room temperature.

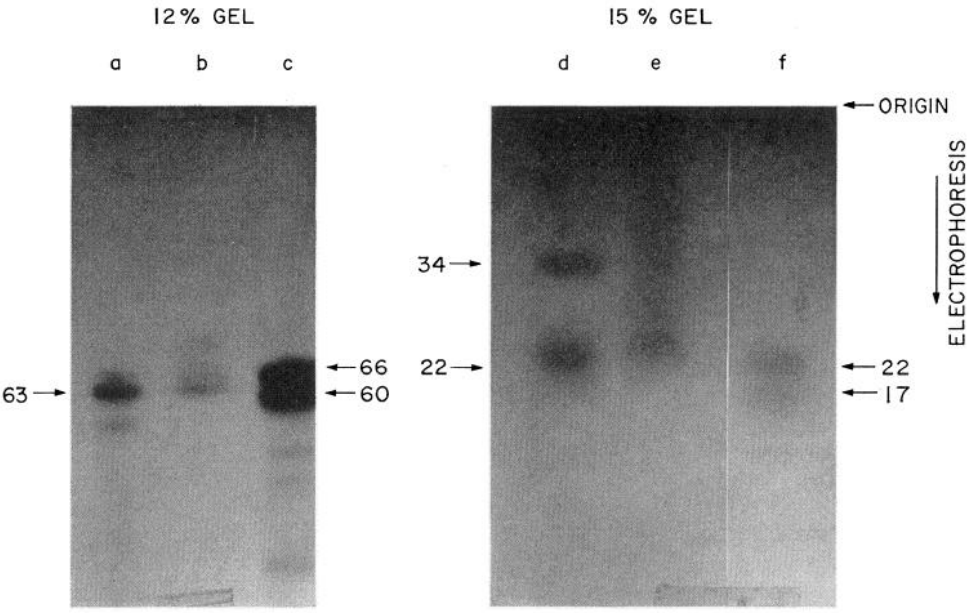

FIG. 12. Gel electrophoresis of the chain-elongated products. The elongated products were separated from the r strand by column chromatography (Fig. 11 for DNA II) or cellulose acetate (for DNA I). The samples were then denatured by boiling in 7 M urea-30% glycerol and subjected to electrophoresis. Channel *a* contained elongated DNA I obtained by using [α-^{32}P]dCTP, dATP, and dTTP; channel *b* contained elongated DNA I obtained by using [α-^{32}P]dATP, dTTP, and dCTP; and channel *c* contained two deoxyribopolynucleotides, 60 and 66 nucleotides in length. Channels *a* to *c* were run on a 12% polyacrylamide gel by Dr. T. Maniatis. Channel *d* contained the mixture of excess and elongated [5′-^{32}P]DNA II; channel *e* contained [5′-^{32}P)]DNA II; and channel *f* contained two deoxyribopolynucleotides, 17 and 22 nucleotides in length. Channels *d* to *f* were run on a 15% polyacrylamide gel.

TABLE VII

Nearest neighbor analysis and Burton degradation analyses of products obtained by elongation of DNA I or DNA II using dTTP, dCTP, and dATP following isolation away from DNA r strand

The numbers in parentheses are the observed molar or product ratios. The conditions for nucleotide incorporation, isolation, and nearest neighbor analysis or depurination are given in "Methods."

a. Nearest neighbor analyses

Experiment	dNTPs used	Radioactivity			
		dAp	dGp	dTp	dCp
		cpm			
1	DNA I + [α-^{32}P]dCTP + dATP + dTTP	599 (1.0)	54	1,323 (2.2)	54
2	DNA I + [α-^{32}P]dATP + dCTP + dTTP	902 (2.7)	110	63	667 (2.0)
3	DNA II + [α-^{32}P]dCTP + dTTP + dATP	6,476 (1.00)	216	13,940 (2.15)	1,130

b. Burton degradation products

Experiment	dNTPs used	Radioactivity	
		*pCpTpTpT*pCp	pT*pCp
		cpm	
1	DNA II + [α-^{32}P]dCTP + dTTP + dATP	11,758 (1.75)	6,678 (1.00)
2	*pCpTpTpTpCp following phosphatase	CpTpTpTpC* 4,844	*P 4,909

urea alongside two synthetic polynucleotides of size 22 and 17 nucleotides (Fig. 12). Only two radioactive products were present, one corresponding to the unchanged DNA II (22 nucleotides long) and the second corresponding to a polynucleotide of about 34 nucleotides. The ratio of radioactivity (655 cpm in DNA II and 340 cpm in the elongated DNA II) was as would have been expected.

DISCUSSION

Using DNA polymerase I to add nucleotides to the 3′ end of synthetic deoxyribopolynucleotides when the latter are annealed to the ϕ80psu$_{III}$ r strand, a start has been made in sequencing the DNA region beyond the C-C-A end of the tyrosine suppressor tRNA. First, the sequence C-C-A itself was found to be present in the tRNA^{tyr} gene, and this result is in agreement with that of Altman and Smith (3). Second, the sequence of the next 12 nucleotides was determined by using a variety of labeling and degradative methods.[4]

It was important to establish that the primers are used by the DNA polymerase specifically at the tRNA sequence site. The 51-nucleotide-long primer (DNA I) was used in a ratio of 1:1 with the r strand and from previous work (2); it would be expected to bind at the tRNA site. The 22-nucleotide-long primer (DNA II) has been shown previously to bind to the r strand at a site additional to the tRNA site. In the present work, the primer was used in a 2-fold excess to the r strand. This seemed necessary in order to obtain nucleotide incorporations equivalent to the r strand. With both DNA I and DNA II, the polymerase-catalyzed additions occurred uniquely at the 3′ end of the tRNA sequence. Thus, the extent and nature of nucleotide incorporations were identical, and the same unique sequence of 12 nucleotide units was obtained.

Finally, the extended products from the 5′-^{32}P-labeled primers were isolated and subjected to electrophoresis in 12 or 15% gels (Fig. 12). With both DNA I and DNA II, the expected extended products (chain length 63 and 34, respectively) were found. Especially with DNA II, where an excess had been used, the second major ^{32}P product corresponded to the unchanged starting material. Thus, there was no evidence of degradation or the formation of a second extended primer with DNA II.

Further application of the present approach to determine the

[4] S. Altman (personal communication) has recently succeeded in isolating an *in vivo* precursor molecule to the suppressor tRNA which has about 12 to 15 extra nucleotides at the 3′ end. Preliminary data indicate that the composition of the extended segment is similar to that which we have reported here.

sequence beyond the 12 nucleotide units is possible in at least two ways. In the first, the primers used in the present work may be extended using cold dATP, dTTP, and dCTP; the extended primers were isolated and reused with "fresh" samples of the r strand. This approach is already giving encouraging results. A variant of this approach would be to synthesize the suitable primers which include the newly discovered sequence of 12 nucleotide units. Work along this line is in progress. Although slow, this approach may give further confirmation of the sequences derived and, in any event, the progressive extension of the DNA duplex corresponding to the precursor $tRNA^{tyr}$ as the sequences become known would be necessary to test when the termination sequences are complete.

A more attractive application of the primer-template approach appears to be the synchronized extension of the primers using all the four labeled deoxyribonucleoside triphosphates. (Radioactive labeling could be introduced as desired.) The chain growth could be studied as a function of time in a manner analogous to that used by Billeter and co-workers for the sequencing of $Q\beta$ RNA (19). The products could be investigated by fingerprinting, partial degradation, and related methods (*e.g.*, by the incorporation of a ribonucleoside 5′-triphosphate in place of the corresponding deoxyribonucleoside 5′-triphosphate (13). Work along these lines is in progress.

Work is also in progress to determine the promoter sequence beyond the 5′ end of the $tRNA^{tyr}$ precursor. In this case, a suitable synthetic deoxyribopolynucleotide corresponding to the 5′-triphosphate end of the tRNA precursor is annealed to the l strand of $\phi 80psu_{III}$ DNA. DNA polymerase-catalyzed nucleotide incorporations then give information on the sequence beyond the 5′ end of the tRNA precursor.

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