Enzymatic synthesis, ligation, and restriction of DNA containing deoxy-4-thiouridine

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ABSTRACT
Phage fd RF I DNA, about 90% substituted by deoxy-4-thiouridine \((s^4T_d)\) in the codogenic strand was synthesized by the simultaneous actions of DNA polymerase I and DNA ligase. While the rate of DNA synthesis was considerably reduced, the yield was not affected in the presence of \(s^4T_dTP\). The conversion of RF II to RF I DNA by DNA ligase was even improved. This effect seems to be related with an altered ratio of affinity of polymerase and ligase for the \(s^4T_d\)-containing substrate. The presence of the base analogue in the DNA was verified independently by chromatographic and spectroscopic methods. The modified genome could be cleaved by restriction endonucleases Hpa II \((C/CGG)\) and Taq I \((T/CGA)\). A number of the fragments produced showed altered mobilities under the conditions of polyacrylamide gel electrophoresis.

INTRODUCTION
Modified nucleic acids have proved to be useful tools for many purposes in molecular biology. For example, a large number of studies on nucleic acid-nucleic acid (e.g., 2-5) and nucleic acid-protein interactions (e.g., 6-15) have been published which made use of modified DNAs. Moreover, site-directed mutagenesis can also be performed by the incorporation of nucleotide analogues into DNA (16) or RNA (17).

At present a limited number of modified nucleotides is available that can be enzymatically incorporated into nucleic acids of natural sequence. One of the positions in DNA not accessible to modification so far was the 4-keto group of thymidine \((T_d)\), which is of particular interest as it is involved in Watson-Crick base pairing.

Enzymatic synthesis of alternating polymers containing deoxy-4-thiouridine \((s^4T_d)\) were reported several years ago.
But difficulties were met in attempts to incorporate the analogue into activated calf thymus DNA (18,20). Since such a DNA is no well-defined template and detailed data on the reaction were not available, we found it worthwhile to re-examine this subject.

We used E.coli DNA polymerase I, $^{3}T_dTP$ and a template of natural origin and known sequence (21), the circular single-stranded DNA of bacteriophage fd. In the presence of DNA ligase we were able to obtain the double-stranded closed circular RF I DNA containing the modified nucleotide in the minus-strand. The DNA or its degradation products were characterized by spectroscopic, electrophoretic and chromatographic methods.

**MATERIALS AND METHODS**

**Enzymes.** E.coli DNA polymerase I and T4 DNA ligase were isolated in this laboratory by H. Müller and R. Frank, respectively, according to standard methods (22,23). E.coli DNA ligase was prepared similar to published procedures (24,25). Specific activity of DNA polymerase I: 8160 units per mg. One unit catalyses the incorporation of 10 $\mu$mol of nucleotides into polyA$_d$T$_d$ in 30 min at 37°C under assay conditions (22). One unit of DNA ligase converts 100 $\mu$mol (nucleotides) of poly(A$_d$T$_d$) into exonuclease III-resistant covalently closed circles in 30 min at 30°C under assay conditions (23,26). Restriction endonuclease Taq I from Thermus aquaticus was a kind gift of Dr. H. Mayer, Stöckheim. Restriction endonuclease Hpa II from Haemophilus parainfluenzae was supplied by Miles Laboratories, Elkhart, Indiana, USA. Micrococcal nuclease from Staphylococcus aureus, spleen phosphodiesterase, DNase I from bovine pancreas (grade I), and snake venom phosphodiesterase were purchased from Boehringer, Mannheim, FRG.

**Substrates.** Viral single-stranded DNA from bacteriophage fd and fd-specific oligonucleotide primers were prepared as previously described (10). Nucleoside triphosphates were purchased from Boehringer, Mannheim, FRG. $[^{3}H]C_dTP$ was supplied by New England Nuclear, Boston, Mass., USA. $[^{32}P]A_dTP$ was from The Radiochemical Centre, Amersham, UK. $^{3}T_dTP$ was synthesized similar to the procedure published by Scheit (27). As judged
from chromatographic analysis and UV absorption spectrum it was
more than 95\% pure.

**DNA synthesis and ligation.** These procedures were performed
as described (15) with the following modifications: Either
C\textsubscript{d}TP (1.6 \times 10\textsuperscript{5} cpm/nmol) or A\textsubscript{d}TP (9.2 \times 10\textsuperscript{5} cpm/nmol) were radio-
labeled, 60 units of DNA polymerase I and 2.5 units (in the pre-

cence of T\textsubscript{d}TP) or 0.9 units (in the presence of s\textsubscript{d}T\textsubscript{d}TP) of T\textsubscript{d}
or E.coli DNA ligase were used. The mixture was incubated at
20°C for 4 h (in the presence of T\textsubscript{d}TP) or 16 h (in the presence
of s\textsubscript{d}T\textsubscript{d}TP). After deproteinization preparative purification of
RF I was performed by acid phenol extraction (28).

**Chromatographic analysis of s\textsubscript{d}T\textsubscript{d}MP incorporation.** DNA syn-
thesis in the presence of [\textsuperscript{32}P]A\textsubscript{d}TP was performed as described
above. The ethanol precipitated pellet was washed with ethanol
and dissolved in 5mM Tris base. The DNA was subsequently de-
graded by St. aureus nuclease and spleen phosphodiesterase as
described by Josse et al. (29). The digests were chromato-
graphed on silica gel thin layer (60 F25\textsubscript{4} from Merck, Darmstadt,
FRG) using ethanol-1M ammoniumacetate, pH 7.5, 7:3 (v/v) as
solvent. The products were detected by autoradiography on
Kodix X-ray films from Kodak.

**UV-spectroscopic analysis of s\textsubscript{d}T\textsubscript{d}MP incorporation.** Spectro-
scopical measurements were performed at room temperature in a
Gilford 2400-S spectrophotometer. RF I DNA was dissolved in 100
mM Tris-HCl, pH 8.0, 10 mM MgCl\textsubscript{2}, 0.2 mM EDTA. Degradation
to mononucleotides was performed in this solution at room
temperature by subsequent incubation with DNase I from bovine
pancreas and snake venom phosphodiesterase (30).

**Fragmentation of s\textsubscript{d}T\textsubscript{d}-substituted DNA.** Hpa II: 2 \mu g of RF I
DNA were incubated in 60 \mu l of 10 mM Tris-HCl, pH 7.5; 10 mM
MgCl\textsubscript{2}; 5 mM KCl; 1 mM DTE; 2.5\% glycerol with 5.5 units of
Hpa II at 37°C for 7 h. Taq I: 2 \mu g of RF I DNA were incubated
as above in 10 mM Tris-HCl, pH 7.5; 10 mM MgCl\textsubscript{2}; 5 mM KCl;
10 mM ME; 5\% glycerol with 15 units of Taq I.

**Gel electrophoresis.** The conditions for agarose (buffer A)
and polyacrylamide gel electrophoresis as well as visualization
and photography of DNA bands habe been described earlier (10,
15). Buffer D for agarose gel electrophoresis contained 40 mM
Tris·HOAc; 5 mM NaOAc; 2 mM EDTA; pH 7.8 (31).

**Velocity sedimentation.** Velocity sedimentations were performed in alkaline 5-20% sucrose gradients. Runs were for 50 min at 50,000 rpm and 15°C in a Beckmann/Spinco SW 60 rotor.Sucrose solutions contained KOH at 0.2 M; Tris·HCl, pH 7.5, at 10 mM; NaCl at 0.5 M; EDTA at 1 mM; Sarcosyl NL 97 (Ciba-Geigy) at 0.075%.

**RESULTS**

**DNA synthesis and ligation.** The template-directed incorporation of s$^3$TP into bacteriophage fd RF DNA was examined. DNA synthesis was catalyzed by E. coli DNA polymerase I. Priming oligonucleotides originated from a DNase digest of fd RF DNA. The conditions for DNA synthesis were almost exactly those used for the 'repair' synthesis of unmodified RF molecules (15). T$^3$TP was replaced by s$^3$TP. T$^4$ DNA ligase (or the E. coli enzyme) was present to allow conversion of nicked circular RF II DNA into covalently closed circular RF I DNA. (For details see Materials and Methods.) The reaction was monitored by incorporation of $[^3H]$UMP or $[^32P]$AMP into acid-precipitable material.

Fig. 1 shows that in the presence of s$^3$TP synthesis started immediately after addition of DNA polymerase, but proceeded at a much lower rate than in the presence of T$^3$TP. Nevertheless the same amount of DNA was obtained in both cases. The reactions reached plateau values after about 1.5 h and 15 h, respectively. In a control assay, when neither T$^3$TP nor s$^3$TP were present, no DNA synthesis was observed (fig. 1).

The intermediates and final products of the reaction were analyzed by agarose gel electrophoresis. Fig. 2a demonstrates that DNA synthesis in the presence of s$^3$TP was initiated on all template molecules and led to covalently closed RF I DNA. The formation of this species was independently verified by a cellulose nitrate filter assay which makes use of the selective denaturation of RF II and RF III DNAs (32,33), and by velocity sedimentation in an alkaline sucrose gradient as shown in fig. 3. Minor amounts of linear full-length double-stranded RF III DNA were found (fig. 2a). As the template contained only traces of linear DNA it seems that the appearance of this
Figure 1: Time course of DNA synthesis catalyzed by DNA polymerase I. At times 5μl- aliquots were withdrawn from the reaction mixture and assayed for the incorporation of $^{3}$H from $[^3H]GTP$ into acid-precipitable material. The reaction was carried out in the presence of $A_dTP$, $G_dTP$, $C_dTP$, and $T_dTP$ (open circles); $A_dTP$, $G_dTP$, and $C_dTP$ (triangles); $A_dTP$, $G_dTP$, $C_dTP$, and $s^H_T_dTP$ (full circles).

species was due to traces of endonuclease contaminations.

Agarose gel electrophoresis shows that only relatively small amounts of nicked circular RF II DNA (being precursors of RF I DNA) were present at any time samples were withdrawn from the reaction mixture. When normal DNA was synthesized under identical conditions considerably more RF II species were observed (fig. 2b). But when the rate of DNA synthesis was decreased by reducing the amount of DNA polymerase I about 3.5-fold, the conversion of RF II species into RF I molecules could be enhanced to about the same amount as observed in the presence of $s^H_T_dTP$ (fig. 2a).

When RF I DNA is synthesized under the conditions described above, a Gauss distribution of molecules with different topological winding numbers is obtained which can be separated by agarose gel electrophoresis in the absence of ethidium bromide (34,35). No significant difference in the electrophoretic pattern of normal and $s^H_T_d$-substituted RF I DNAs was observed (fig. 2c).

During the isolation of RF I DNA we noticed that considerable amounts of this species were lost by conversion to
Figure 2: Analysis of the products of DNA synthesis by agarose gel electrophoresis. Migration is from top to bottom. a: Synthesis in the presence of s^T dTP. 3μl-samples were withdrawn at zero time (lane 1), 1 h 35 min (lane 2), 5 h 35 min (lane 3), 15 h 45 min (lane 4) and electrophoresed in buffer A containing ethidium bromide. b: Synthesis in the presence of T_dTP. 2 h after the amount of acid-precipitable radioactivity had reached a plateau the products were analyzed as above. DNA synthesis was catalyzed by 60 units/ml (lane 1) or 18 units/ml (lane 2) of DNA polymerase I. c: End products electrophoresed in the absence of ethidium bromide (buffer B), s^d-substituted DNA (lane 1) and normal DNA (lane 2).

RF II. Further examination of this phenomenon revealed that nicking occurred almost exclusively during ethanol precipitation and/or resolution of the pellet with a vortex mixer. This seems to indicate an increased susceptibility of the modified DNA to mechanical forces.

As the T_d-analogue displays an absorption maximum at 335 nm, the s^T_d-content of the isolated RF I DNA was examined by UV spectroscopy. The absorption spectrum clearly indicated the presence of the modified base (fig. 4). In order to quantitate the amount of s^T_dMP present in the DNA preparation the molecules had to be degraded monomers, as the percentage of hyperchromicity of this DNA was unknown. This was done at pH 8.0 by the subsequent actions of DNase I and snake venom phosphodi-
Figure 3. Analysis of the products of DNA synthesis by velocity sedimentation in an alkaline sucrose gradient. The fractions were assayed for acid-precipitable radioactivity. 

- a: DNA synthesized in the presence of $s^{32}$TpTP. 
- b: DNA synthesized in the presence of TpTP.

Esterase treatment led to a shift of the short wavelength maximum from 255 nm to 259 nm. No significant shift of the long wavelength maximum was observed. An increase in absorbance of 62% and 115% at 260 nm and 335 nm, respectively, was found. This yields an absorbance ratio $A_{260}/A_{335}$ of 4.35. The theoretical value was calculated using the following data for the molar absorption coefficients of the nucleotides: 

- a) at 260 nm: $A_d$: 15400, $G_d$: 12010, $C_d$: 7050, $T_d$: 8400, and $s^{32}T_d$: 2500 (19);
Figure 4: Ultraviolet absorption spectrum and hyperchromicity of fd RF I DNA synthesized in the presence of s^TTP. The DNA was dissolved in 100 mM Tris-HCl, pH 8.0; 10 mM MgCl₂, 0.2 mM EDTA. A, before, and B, after degradation to mononucleotides.

b) at 335 nm: zero for the normal nucleotides and 21000 for s^T_d (19). The values for the base composition of fd RF DNA were derived from the known sequence of the plus-strand: 34.5% A_d, 24.6% T_d, 20.7% C_d, 20.2% G_d. This leads to a theoretical absorbance ratio \( \frac{A_{260}}{A_{335}} = \frac{3.95}{4.53} \approx 0.88 \). It follows that a value of 4.53 corresponds to an 88\% s^T_d TP-substitution of the minus-strand.

To independently prove the s^T_d MP content of the molecules DNA synthesis in the presence of \([α-32P]_d TP\) was performed. The product was degraded by the subsequent actions of micrococal
nuclease and spleen phosphodiesterase to yield labeled 3'-nucleotides. These were chromatographed on a silica gel thin layer. The digest of unmodified DNA was run as a control. With ethanol-1 M ammonium acetate, pH 7.5, 7:3 (v/v) as solvent $^{4}$T_{d}MP and T_{d}MP were separated from each other and from the other nucleotides. As shown in fig. 5, DNA synthesized in the presence of $^{4}$T_{d}TP in fact contained a new component which was not present in the normal DNA and had the chromatographic properties of $^{4}$T_{d}MP (unlabeled 5'-$^{4}$T_{d}MP run as additional control, not shown). Minor amounts of T_{d}MP, presumably resulting from hydrolysis of the 4-keto-group, were detected. They figured up to about 14% as quantified by Cerenkov counting and densitometric evaluation. This is in good agreement with the spectroscopic analysis.

Restriction endonuclease cleavage. $^{4}$T_{d}-containing fd RF DNA

Figure 5: Chromatographic analysis of mononucleotides after hydrolysis of DNA. DNA was synthesized in the presence of $^{[\alpha-32p]}$dATP. It was digested subsequently by nuclease from St. aureus and spleen phosphodiesterase to yield 3'-N_{d}MPs. The products were chromatographed on silica gel thin layer. The appropriate 5'-N_{d}MPs were run as markers (not shown). Lanes 1 and 2 show DNA synthesized in the presence of T_{d}TP or $^{4}$T_{d}TP, respectively. O=origin.
was incubated with restriction endonucleases Hpa II and Taq I, normally recognizing the sequences \((C/CGG)_d\) and \((T/CGA)_d\), respectively. The products were analyzed on 3.5% polyacrylamide/7M urea gels. As shown in fig. 6a, the modified DNA was cleaved not only by Hpa II which has only C and G in its recognition site, but also by Taq I normally cutting next to T. It was observed that the rates of cleavage by Hpa II were reduced by the DNA modification. The amount of enzyme had to be enhanced about 5-fold to achieve complete cleavage within the time necessary to fragment unmodified DNA. At different Hpa II sites the cleavage rates were different. This effect was also observed for the restriction of unmodified fd RF DNA by Hpa II (our unpublished results).

\(s^t_d\) substitution led to significantly altered electrophoretic mobilities of certain DNA fragments in polyacrylamide gels (fig. 6). The modified Hpa II-fragments D and E (0.652 and 0.648 kb) comigrated under the conditions applied, whereas the unsubstituted fragments were clearly separated. The same holds for Taq I-fragments G and H (presumably 0.381 and 0.357 kb). On

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**Figure 6**: Cleavage of fd RF DNA by restriction endonucleases. The products were analyzed by electrophoresis in a 3.5% polyacrylamide gel. Migration is from top to bottom. XC = xylene cyanol. a: s^t_d-substituted RF DNA cleaved by Taq I (lane 1) or Hpa II (lane 2). b: Normal RF DNA cleaved by Hpa II. c: Normal RF DNA cleaved by Taq I.
the other hand $s^4 T_d$ incorporation improved the separation of the Hpa II-fragments G and H ($0.454$ and $0.381$ kb). These effects did not correlate with the $s^4 T_d$-content of the fragments, but obviously were sequence specific.

**DISCUSSION**

Several years ago $s^4 T_d$TP had first been synthesized and tested as substrate for DNA polymerases (18-20). It was found that templates of strictly alternating sequences like poly($A_d T_d$) and poly($A_d C_d$) were able to direct incorporation of $s^4 T_d$ into the complementary strand. Using the homopolymer template poly($A_d$), however, Lezius reported inhibition of DNA synthesis in the presence of a 20-750-fold excess of $T_d$TP over $s^4 T_d$TP. This effect was explained by the assumption that incorporation of $s^4 T_d$ inhibits further primer elongation by DNA polymerase I. With activated calf thymus DNA only little incorporation of $s^4 T_d$ was observed when $T_d$ or $s^4 T_d$ (no discrimination between these two possibilities was given) were the preceding nucleotides.

We re-examined the incorporation of $s^4 T_d$ into DNA replacing a merely defined substrate like calf thymus DNA by unique template molecules of known sequence. Using the circular single-stranded genome of bacteriophage fd to direct nucleotide incorporation, full-length complementary strand synthesis could not only be checked by chain-length determination but even more exactly by the formation of covalently closed circular RF I molecules in the presence of DNA ligase. We were able to show that in the presence of $s^4 T_d$TP DNA synthesis by E.coli DNA polymerase I was considerably retarded, but not completely inhibited. It was initiated on all template molecules and led to the formation of full-length complementary strands in virtually all cases. The newly synthesized DNA was shown to be about 90% substituted by $s^4 T_d$.

Inspection of the template sequence shows that it contains 5 $(A_d)_6^-$, 12 $(A_d)_5^-$, 33 $(A_d)_4^-$, 79 $(A_d)_3^-$, and 229 $(A_d)_2^-$ stretches. This indicates that $s^4 T_d$-sequences can in fact be synthesized by DNA polymerase I.

In our opinion two major reasons could account for the dis-
crepancies between our results and the earlier reports mentioned above. First, a homopolymer pair like poly(A$_d$)·poly(T$_d$) probably is structurally different from DNA. Hence, results obtained with this system might not be valid for DNA. Second, nuclease contamination of the DNA polymerase might be a critical point. It was found especially in restriction endonuclease reactions that the modified DNA was extremely susceptible to contaminating exo-nuclease activities. Furthermore we formerly observed that U$_d$-containing DNA was so rapidly degraded by exonuclease contaminations of DNA polymerase I preparations that we were not able to obtain RF I molecules in the presence of DNA ligase.

It seems likely that at least under certain conditions the conformation of sT$_d$-containing DNA differs from that of normal DNA. Although the A$_d$·sT$_d$ base pair seems to be of the Watson-Crick type (36), replacement of the NH···O bond (2.9 Å) of the A$_d$·T$_d$ pair by a NH···S bond (3.3 Å) (37) should give rise to a distortion of the modified base pair. In fact X-ray diffraction studies on poly(A$_d$·sT$_d$) fibers revealed a deviation of the A$_d$·sT$_d$ pair from planarity (Saenger, W., personal communication). Furthermore it was noticed that sT$_d$-substituted DNA fragments show altered electrophoretic mobilities. As these effects were not proportional to the sT$_d$-content of the respective fragments, they cannot be explained by an altered net charge alone, but seem to reflect sequence-specific structural changes. Similar observations were also made for other base analogues (15). The enhanced susceptibility of the modified RF I DNA to mechanical forces might indicate an unusual conformational strain.

Another interesting difference between normal and sT$_d$-containing DNA is the magnitude of the hyperchromic effect. At 260 nm the hyperchromicity of the modified DNA totaled to about 62%. For normal DNA of similar base composition values around 80% were observed at a similar pH (38, and our own observations). This indicates that the incorporation of sT$_d$ leads to a significant reduction of the stacking interactions of the normal nucleotides. This may be explained by a partially reduced overlap of p-orbitals of the heterocyclic bases due to the deviation of the A$_d$·sT$_d$ base pair from planarity. The hyperchromicity at
335 nm indicates that the nucleotide analogue is involved in base stacking. The value of 115% is relatively large. It is rather small, however, compared to the respective value of 173% observed for poly(A₁₉₆) (19). Interestingly the hyperchromic effect of this alternating polymer was also larger at 260 nm (80%).

The rate of primer elongation by DNA polymerase I in the presence of s₄T₄TP was significantly reduced. It is not known if this effect reflects the differences between T₄ and its analogue in the interaction between the enzyme and the N₄TP, the enzyme and the base pair or the enzyme and the primer terminus. There is some evidence that at least the third alternative might play a role. In the presence of DNA ligase s₄T₄-containing DNA is sealed faster than normal DNA. The ligation of the normal substrate can be improved by lowering the polymerase concentration. This seems reasonable as both enzymes compete for nicks (ligation vs. nick-translation). Obviously the ratio of binding of polymerase and ligase is lowered for the modified DNA. It follows that the affinity for nicks of at least one of the two proteins is changed with the s₄T₄-substituted substrate.

Our studies on fragmentation of the modified RF I DNA show that not only a restriction endonuclease recognizing sequences without T₄, like Hpa II, is able to specifically cleave this substrate, but that also an enzyme like Taq I, which normally cuts next to T₄, generates the fragments expected. So in principle the advantages of site-specific DNA cleavage can also be applied to s₄T₄-containing DNA.

The findings that the modified DNA is fragmented considerably more slowly by Hpa II, and that individual Hpa II-sites on fd RF DNA are cleaved at different rates, confirm our earlier observations that sequences outside the Hpa II-recognition site display a remarkable influence on the enzymatic activity (15).

Based on the results presented here it may be expected that DNA sequences in general are accessible to modification by s₄T₄. It should also be possible to site-specifically fragment the products. They may be used in studies on DNA-DNA and DNA-protein interaction. As it was shown that the analogue can be attacked by several reagents under rather mild conditions (e.g.,
39-41), they should also be versatile probes or precursors for further DNA modifications.

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