Chemical and enzymatic analysis of covalent bonds between peptides and chromosomal DNA

Benediktas Juodka, Marita Pfutz and Dieter Werner
Department of Biochemistry and Biophysics, Vilnius University, Vilnius, Lithuania and Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, FRG

Received September 23, 1991; Revised and Accepted November 14, 1991

ABSTRACT
DNA from Ehrlich ascites tumor (EAT) cells and from human placenta was examined for covalent bonds between hydroxy amino acid residues in peptides and nucleotide phosphate groups. The residual proteinaceous material in highly purified DNA was radiolabelled with \(^{125}\)Iodine and the linking-groups between peptides and nucleotides released by combined protease and nuclease treatment were investigated with respect to their chemical and enzymatic stabilities. The residual nucleotide(s)-peptide(s) fraction from DNA isolated after prolonged alkaline cell lysis and phenol extraction contains mainly alkali and acid-stable but phosphodiesterase-sensitive peptide-nucleotide complexes which indicates phosphodiesterases between tyrosyl residues in peptides and nucleotide phosphates. In contrast, the linking-group fraction from DNA isolated under native conditions contains additional peptide components, (a) Phospho-peptides that co-purify with DNA but that are not covalently bound to nucleotides. (b) A fraction of peptides that is released from nucleotides by alkali in a time and concentration-dependent reaction. Evidence is presented indicating that the latter fraction involves phospho-triesters between hydroxy amino acid residues in peptides and internucleotide phosphates. The phosphodiesterases between hydroxy amino acids and nucleotide phosphates representing the predominant class of peptide-nucleotide complexes in alkali-denatured DNA are most likely side products of peptide-nucleotide phospho-triester hydrolysis.

INTRODUCTION
To rid the polyanionic polymer DNA from physiologically and unspecifically adhering polycationic proteins is of interest since the discovery of DNA [1]. Numerous procedures were developed to isolate and to purify DNA, however, it is well known that a persistent fraction of proteinaceous material is inevitably copurified with chromosomal DNA. These tight DNA-polypeptide interactions are of interest because DNA-polypeptide complexes with higher stabilities than nucleosomes are considered by many authors to be involved in the folding of the genome in higher order structures and in the determination of the cell type-specific patterns of active genes. However, at present, the significance of these protein-DNA complexes and the nature of the chemical bonds involved are not yet completely understood and deserve further elucidation.

The method for isolation of chromosomal DNA designed by Gross-Bellard et al. [2] releases rather pure DNA. However, despite of repeated treatments with SDS / proteinase K and phenol, DNA isolated by this or similar procedures still contains residual proteinaceous material. For example, additional treatment with alkaline buffers releases another fraction of peptides [3] and even DNA isolated by prolonged cell lysis with strong alkali retains distinct polypeptides that are only released after degradation of the DNA [4]. These findings and other results indicating that chromosomal DNA contains structures becoming more alkali-sensitive after prolonged protease treatment [5, 6] were regarded as indications for covalent bonds between (polypeptides and nucleotides in chromosomal DNA. The first rigorous analysis of linking-groups between peptides and nucleotides in chromosomal DNA has been performed with DNA from Ehrlich ascites cells isolated by prolonged alkaline cell lysis and phenol extraction [7]. The linking-groups isolated from this DNA by combined protease and nuclease treatment remained unchanged in alkali and acid, however, they could be cleaved by phosphodiesterases [7]. Since phosphodiesterases are rather specific for nucleotide ligands in phosphodiesterases while being unspecific with respect to the hydrolytic release of the second ligand [8–11] it was concluded that alkali-denatured DNA contains phosphodiesterases between hydroxy amino acid residues in peptides and nucleotide phosphates [7]. Using the same rigorous approach, other authors could detect phosphodiesterases of this type also in DNA from other sources [12, 13].

In this study we investigated whether chromosomal DNA isolated under native conditions contains other or additional covalent complexes between hydroxy amino acids in peptides and nucleotides that cannot exist in alkali-treated DNA because of the alkali sensitivity of the bonds involved.

* To whom correspondence should be addressed
MATERIALS AND METHODS

Isolation of DNA

Alkali-denatured DNA was isolated from Ehrlich ascites cells by prolonged alkaline cell lysis as described previously in detail [4]. Briefly, freshly harvested cells were suspended in EDTA buffer, mixed with 1 vol of 1 M NaOH and kept at room temperature for several hours which was followed by phenol extractions and ethanol precipitation of the aqueous phase. Native DNA was isolated from Ehrlich ascites cells by the method described by Gross-Bellard et al. [2]. This method includes cell lysis in the presence of SDS / 50 μg per ml of proteinase K (Merck), repeated phenol extractions, digestion with ribonucleases, a second proteinase K digestion as described, repeated phenol extractions and finally precipitation with ethanol. DNA from human placenta tissue was isolated from liquid nitrogen-frozen and powdered tissue [14] by the method of Gross-Bellard et al. [2] as described above.

Radiolabelling of residual proteins in DNA preparations with 125Iodine

This method has been described previously in detail [7]. Briefly, 10 mg portions of DNA (200 OD260 units) precipitated with ethanol were suspended in 1.4 ml of iodination buffer composed of 3 g of urea, 100 mg of SDS, 3.5 ml of 0.5 M Tris-HCl, pH 7.6 and mixed with 2 mCi of Na125I (17.4 Ci per mg, NEN) and 50 μl chloramin T solution (12 mg / ml of water). After 30 min at room temperature, Iodine was reduced by addition of 100 μl of β-mercapto ethanol. DNA was collected together with associated radiolabelled proteins by repeated (5x) precipitations with ethanol.

Isolation of nucleotide(s)-peptide(s) fractions

The pellets of the DNA-protein fractions were digested with 6 mg proteinase K per ml (16 h, 37°C, 0.25% SDS, 20 mM EDTA, 150 mM NaHCO3, pH 8.0) which was followed by repeated (5x) ethanol precipitations. Denatured DNA was precipitated from alkaline (0.5 M NaOH) solutions, native DNA was suspended between ethanol precipitations in Tris-EDTA buffer. The residual DNA-peptide fraction was finally precipitated and the pellets were dissolved in nuclease buffer (46 mM sodium acetate, 10 mM magnesium sulfate, 3.5 mM zink sulfate, pH 5.0) and digested (16 h, 37°C with 100 mg per ml of DNase I (RNase-free, Boehringer) and 50 × 103 units per ml of S1 nuclease (Boehringer).

Chemical and enzymatic treatments

Aliquots of the residual nucleotide(s)-peptide(s) fractions were mixed with chemical agents (e.g. NaOH, HCI) to give the final concentrations indicated in the figure legends or they were adjusted to the optimal buffer conditions before digestion (at 37°C) with the following enzymes: Alkaline phosphatase from calf intestine (150 U×ml−1, Boehringer), snake venom phosphodiesterase (oligonucleate-5′-nucleotidohydrolase, EC 3.1.15.1, PDE I, 0.4 U×ml−1, Sigma) and spleen phosphodiesterase (oligonucleate-3′-nucleotidohydrolase, EC 3.1.16.1, PDE II, 4 U×ml−1, Boehringer). The phosphodiesterase-containing culture broth of S.lividans (PRYE1) was kindly supplied by M.K. Speedie, Baltimore. This enzyme was assayed under the conditions described in the literature [15]. A DNA sequencing reagent kit from NEN (NEK-010) was used to perform the chemical cleavage reactions reactions. The prescrip-

Radiochromatography

Free peptides released from DNA and nucleotide complexes were detected by means of chromatography on cellulose TLC sheets (Merck) using citrate-buffered phenol [7] as solvent. Radiochromatograms were scanned by means of a TLC linear analyzer (LB 2820, Berthold) attached with a multi channel analyzer (Silena). Ordinates reflect relative counts per channel.

RESULTS

General procedure

The general procedure designed previously to isolate and to examine the linking-groups between hydroxy amino acids in peptides and nucleotides in chromosomal DNA isolated by strong alkali [7] proved as well appropriate for the analysis of the linking-groups released from native DNA. It involves the steps shown in Scheme I. It is based on the radioiodination of residual proteins co-purifying with DNA, the isolation of the linking-groups by combined protease and nuclease digestion and the identification of the chemical bonds involved in the residual peptide-nucleotide complexes by investigation of their stabilities and sensitivities during chemical and enzymatic treatments. The radiochromatographic procedure applied to distinguish between free and nucleotide-bound peptides takes advantage of the mobility of amino acids, peptides and oligopeptides in citrate-buffered phenol on cellulose TLC sheets and the immobility of their phosphate and nucleotide(s) derivatives in this solvent system [7].

Scheme I
Peptide-nucleotide linking-groups in DNA isolated by alkaline cell lysis

During the prolonged proteolytic digestion of denatured and radioiodinated DNA most of the protein-specific radiolabel is released from DNA and can be extracted with 70% ethanol. The extracted material migrates on TLC sheets developed with citrate-buffered phenol as expected for amino acids and peptides that are not modified by phosphates or nucleotides (Fig. 1). However, a significant portion (~12%) of the radiolabel originally associated with radioiodinated DNA before proteolysis coprecipitates with DNA and remains associated with DNA at the origins of chromatograms (Fig. 1). These peptides are also not released by chromatography after prolonged digestion of this complex with nuclease.

The complexes retaining ~12% of the peptide-specific radiolabel at the origins of chromatograms are rather stable in alkali and acid (Fig. 1). Even harsh chemical treatments release only traces of the radiolabelled peptides, however, the complexes are cleaved by phosphodiesterases resulting in migrating peptides (Fig. 1). The chemical stabilities of the radiolabelled complexes and their substrate characteristics are consistent with the properties of covalent linking-groups composed of nucleotide-5′-(O4-tyrosyl)-phosphodiesters and nucleotide-3′-(O4-tyrosyl)-phosphodiesters. This conclusion is justified because the phosphodiesterases of other hydroxy amino acids are more alkali-sensitive than those of tyrosine [16–18]. Moreover, it is well established that snake venom phosphodiesterase (PDE I) and calf spleen phosphodiesterase (PDE II) cleave phosphodiesterase between nucleotides and non-nucleotide ligands including those between nucleotides and L-tyrosine [8–11].

Figure 1. Radioscans of chromatograms showing the chemical and enzymatic stabilities of peptide-nucleotide linking-groups contained in the nucleotide(s)-peptide(s) fraction from Ehrlich ascites cell DNA isolated by prolonged alkaline cell lysis and phenol extraction. Following digestion of the radiolabelled DNA-polypeptide fraction with proteinase K a significant amount of radiolabelled peptides remain associated with DNA at the origin (1) while proteolytic cleavage products extracted with ethanol migrate (2). The residual radiolabelled peptides associated with DNA (1) are also retained at the origin after digestion with nuclease (3). Only traces of free peptides are released from this complex by harsh treatments with NaOH (0.1 M, 2 h, 56°C, (4)) and HCl (1 M, 2 h, 37°C, (5)), however, free peptides are released after incubation with PDE I (6), PDE II (7) and PDE I plus PDE II (8).

Figure 2. Radioscans of chromatograms showing the chemical and enzymatic stabilities of peptide-nucleotide linking-groups contained in the nucleotide(s)-peptide(s) fractions from DNA of Ehrlich ascites cells (EAT) and from human placenta isolated under non-denaturating conditions. Both, the EAT and the placenta-derived DNA remains associated with significant amounts of radiolabelled peptides after prolonged digestion with proteinase K (1) while the amino acids and peptides extracted with ethanol migrate (2). The results obtained with radioiodinated DNA from EAT and from placenta were identical. Only the results with placenta DNA are shown in (1) and (2). The peptides co-precipitating with DNA are also not chromatographically released after prolonged nuclease digestion (not shown, chromatographic distribution as in (1)). Treatment of the nucleotide(s)-peptide(s) fraction from EAT (3–5) and placenta (6–8) with alkaline phosphatase (3, 6), alkali (4, 7) or first with phosphatase followed by alkali (5, 8) point to three different complexes: (A) the phosphatase-sensitive complex, (B) the alkali-sensitive complex and (C) the phosphatase and alkali-insensitive complex.
Peptide-nucleotide linking-groups in DNA isolated under non-denaturating conditions

The DNA-peptide fractions derived from native DNA contain about 40% of the $^{125}$Iodine label co-precipitating with the DNA after radioiodination which points to additional DNA-associated protease-stable peptides in native DNA that are not present in the denatured DNA-derived fractions. While the denatured DNA-derived nucleotide(s)-peptide(s) fraction consists essentially of radiolabelled peptides in phosphodiester linkages (Fig. 1), the corresponding fraction from native DNA contains three types of peptide-nucleotide complexes differing in their chemical and enzymatic stabilities.

A. The phosphatase-sensitive complex. A portion of the radiolabelled peptides contained in the nucleotide(s)-peptide(s) fraction from native DNA is chromatographically released after incubation with alkaline phosphatase (Fig. 2). This peptide fraction is not considered to be bound to nucleotides by covalent bonds because there exists no hypothetical bond between peptides and nucleotides that could be cleaved by phosphatase. Apparently, the peptides released by phosphatase derive from phosphopeptides that co-precipitate with DNA and that do not migrate in citrate-buffered phenol unless the phosphate groups are eliminated. The phosphatase-sensitive component can be removed from the nucleotide(s)-peptide(s) fractions by phosphatase treatment and ethanol extraction (not shown) while the precipitate contains other complexes described below.

B. The alkali-sensitive complex. A second portion of residual peptides is chromatographically released from the nucleotide(s)-peptide(s) fraction after alkali treatment (Fig. 2). The release of this peptide fraction is time and alkali concentration-dependent which points to the cleavage of covalent bonds (Fig. 3). This alkali-sensitive complex does not consist of alkali-labile phosphodiester linkages between hydroxy amino acids and nucleotides because the fraction of peptides released by alkali is significantly larger (Figs. 2 and 3) than the amount of phosphodiesterase-sensitive material present in the native nucleotide(s)-peptide(s) fraction (Fig. 5).

C. The phosphatase and alkali-stable complex. After phosphatase and alkali treatment the native DNA-derived nucleotide(s)-peptide(s) fractions still contain residual peptides that are neither released by phosphatase nor by alkali (Fig. 2). The phosphodiesterase sensitivity of this component (Fig. 4) shows that it is structurally identical with the alkali-stable phosphodiester linkages between peptides and nucleotides that are the predominant complexes in the nucleotide(s)-peptide(s) fraction of DNA isolated by prolonged alkaline cell lysis (Fig. 1).

It appears that a portion of the phosphodiester linkages in the complex C arise from hydrolysis of the alkali-sensitive complex B. This is clearly evident if the placenta DNA-derived nucleotide(s)-peptide(s) fraction is considered. Although quantitative interpretations of radiochromatograms are difficult, it appears that the amount of peptide-nucleotide phosphodiester linkages in the placenta DNA-derived fraction is larger after alkali treatment.
(Figs. 2 and 4) than before alkali treatment (Fig. 5). This suggests that a portion of the complex B is hydrolyzed in alkaline solution resulting in alkali-stable peptide-nucleotide phosphodiesters.

The alkali-induced increase of phosphodiesters is less evident if the nucleotide(s)-peptide(s) fraction from EAT DNA is considered because this fraction contains low but significant amounts of phosphodiesterase-sensitive material before alkali treatment (Fig. 5). At present, there is no conclusive explanation for the difference in the amounts of phosphodiesterase-sensitive complexes in placenta and EAT DNA. It is unknown whether DNA of EAT cells contains a larger fraction of these sensitive complexes per se or whether these larger amounts are formed during hydrolytical processes during the isolation or storage of DNA.

It is evident that only a portion (~20%) of the alkali-labile material of complex B is converted into the alkali-stable phosphodiesters (Fig. 2). This implies that the native structure is cleaved during hydrolysis in two ways. One major reaction results in free peptides migrating in the chromatographic system and a side reaction releases alkali-stable phosphodiesters that are retained at the origins of the chromatograms (Fig. 2).

**Evidence for a phospho-triester structure in the alkali-sensitive complex B**

There is evidence indicating that the alkali-sensitive complex B contains phospho-triesters between hydroxy amino acid residues in peptides and internucleotide phosphate groups in DNA.

**A. Study of a model compound.** Studying the chemically prepared model compound deoxythymidilyl-(3'-5')-3'-O-acetyl-deoxythymidine-(P1-O)-L-tyrosine ethyl ester [unpublished] it was found that this phospho-triester is rather stable in acid but quickly hydrolyzed in alkaline solution. As judged from the intensity of UV adsorbing spots on chromatograms (not shown) this hydrolysis occurs with high preference between the tyrosyl ligand and the internucleotide phosphodiester group (>90%). However, a small amount of free thymidine is also produced during alkaline hydrolysis which indicates that at least a small portion of internucleotide ester bonds are cleaved resulting in thymidine-3'-(respectively 5')-(O4-tyrosyl)-phosphodiesters. This indicates that phospho-triesters between tyrosine and internucleotide phosphodiester groups show the chemical characteristics of the alkali-sensitive component B contained in the nucleotide(s)-peptide(s) fraction from DNA isolated under non-denaturating conditions.

**B. Maxam–Gilbert reactions.** The mechanisms of base-specific chemical cleavage reactions [19] predict that peptides bound to internucleotide phosphates of purine and pyrimidine dinucleotides are released as phospho-peptides (Scheme II). As shown in Fig. 2, phospho-peptides can be identified by their immobility in the chromatographic system and—for phosphatase treatment—by their migration. This analytical approach was used to investigate whether phospho-peptides are newly formed during the base-specific cleavages. The nucleotide(s)-peptide(s) fractions were first submitted to prolonged digestion with alkaline phosphatase and only the phosphatase-stable material recovered by ethanol precipitation was submitted to the purine and pyrimidine dinucleotide analysis.

![Figure 5](image_url)

**Figure 5.** Radioscans of chromatograms showing the levels of phosphodiesters in native EAT (1–3) DNA and placenta (4–6) DNA-derived nucleotide(s)-peptide(s) fractions before alkali treatments. The fractions from EAT and placenta were first submitted to a prolonged digestion with alkaline phosphatase. The phosphatase-insensitive material was collected by ethanol precipitation and chromatographically controlled for the quantitative release of the phosphatase-sensitive fraction (1, 2). Other aliquots were digested with phosphodiesterases and submitted to the chromatographic procedure (2, 3 and 5, 6). It should be noted that the amount of phosphodiesterase-sensitive material in the placenta DNA-derived fraction is clearly smaller than that observed after alkali-treatment of this fraction (see Figs. 2 and 4).
pyrimidine-specific cleavage reactions. After hydrazine / piperidine or formic acid / piperidine treatment no freely migrating peptides could be chromatographically detected (Fig. 6), however, additional digestion with phosphatase released a significant portion of radiolabelled peptides (Fig. 6).

The mechanisms of base-specific chemical cleavage reactions [19] also predict that peptides bound to internucleotide phosphates of purine-pyrimidine dinucleotides remain attached to at least one nucleotide (Scheme II). Thus, Maxam—Gilbert reaction-induced increases in the amounts of peptide-nucleotide phosphodiesterase-sensitive structures are as well indicative for phospho-triesters in the native structure. Application of the radiochromatographic approach for the detection of peptide-nucleotide phosphodiesterases revealed that significant amounts of phosphodiesterase-sensitive structures are formed during the base-specific chemical cleavage reactions (Fig. 6).

The results obtained by means of Maxam—Gilbert reactions show that the nucleotide(s)-peptide(s) fractions from EAT and placenta contain structures composed of hydroxy amino acid residues in peptides esterified with internucleotide phosphate groups. Although radiochromatographic analyses of this type are difficult to quantitate, the results point to a cell type-dependent preference of the locations of the phospho-triesters. For example, after hydrazine / piperidine treatment of the placenta DNA-derived fraction significant amounts of phosphatase-sensitive material is released and migrates on the chromatograms (Fig. 6) while almost no phospho-peptides are detected after this treatment in the nucleotide(s)-peptide(s) fraction from EAT DNA (not shown). In contrast, more phosphatase-sensitive material is generated by treatment of the nucleotide(s)-peptide(s) fraction from EAT DNA by formic acid / piperidine while this treatment could not release significant amounts of phosphatase-sensitive material from the corresponding fraction from placenta DNA (not shown). This indicates that in placenta DNA the complexes reside with some preference between pyrimidine nucleotides whereas in Ehrlich ascites cell DNA the complexes are located between purine nucleotides with higher probability.

C. Experiments with phospho-triesterase-containing culture broth.

A class of organophosphorus acid anhydrases has been described exhibiting a broad specificity for phospho-trieste substrate hydrolysis [20—22]. Plasmids encoding proteins with such activities have been subcloned and expressed in different prokaryotic systems [20—22]. In Streptomyces lividans, the plasmid pRYEl-encoded phosphotriesterase is expressed at high levels as a secreted soluble enzyme [15, 22]. A culture broth containing 18 U per ml of Parathion-hydrolyzing activity was used to test whether phospho-triesters in the nucleotide(s)-peptide(s) fractions could serve as substrates. Results of such experiments are shown in Fig. 7. Incubation of the nucleotide(s)-peptide(s) fractions with this phosphotriesterase-containing culture broth released significant amounts of radiolabelled peptides. It

![Figure 6](image6.png)

**Figure 6.** Radiocharts of chromatograms showing the enzymatic analysis of the reaction products formed during purine and pyrimidine-specific Maxam—Gilbert cleavage reactions. The nucleotide(s)-peptide(s) fractions from EAT and from placenta DNA were first submitted to prolonged digestion with alkaline phosphatase and chromatographically controlled for the quantitative release of the phosphatase-sensitive fraction as shown in Fig. 5. The residual fractions depleted of the phosphatase-sensitive peptides from EAT (1—4) and placenta (5—8) were submitted to purine and pyrimidine-specific cleavage reactions. Panels (1) and (5) show the non-migrating radiolabelled peptides after the cleavage reactions indicating that on this level the peptides are still associated with components preventing their migration in the chromatographic system. Panels (2) and (6) show that new phosphatase-sensitive material was formed during the cleavage reactions. It appears also that the phosphodiesterase-sensitive material was increased during the cleavage reactions (3, 7 and 4, 8, compare Fig. 5). It should be noted that Maxam—Gilbert reactions were designed to introduce rare cuts into DNA strands. Even after prolonged incubations with the cleaving agents (5×, see Methods) it cannot be expected that all bases are released. Consequently, only a fraction of radiolabelled peptides can be expected to become phosphatase or phosphodiesterase sensitive after the cleavage reactions.

![Figure 7](image7.png)

**Figure 7.** Radiocharts of chromatograms showing the effects of incubations of the nucleotide(s)-peptide(s) fractions with phosphotriesterase-containing culture broth of *S. lividans* (pRYEl). Panel (1) shows the chromatographic distribution of the radiolabelled peptides in the placenta DNA-derived nucleotide(s)-peptide(s) fraction before the incubation, and panel (2) exhibits the distribution of radiolabelled peptides after the incubation with the culture broth. Similar results were obtained with the fraction from EAT DNA (not shown). Panel (3) shows the distribution of radiolabelled peptides after incubation of the nucleotide(s) peptide(s) fraction from EAT that was first depleted of the phosphatase-sensitive material as described in the legend of Fig. 5.
could be argued that this release of radiolabelled peptides from the complex B may be due to a phosphatase activity in the culture broth. However, phosphatase-pretreated nucleotide(s)-peptide(s) fractions collected by ethanol precipitations could as well serve as substrates (Fig. 7). It is unlikely that the culture broth contains a protease cleaving residual oligopeptides in the complex because the peptides contained in the residual nucleotide(s)-peptide(s) fraction survived the prolonged digestion with protease K during the general procedure applied to isolate the nucleotide(s)-peptide(s) fractions.

It is evident that the fraction of radiolabelled peptides released by the phosphotriesterase-containing broth (Fig. 7) is smaller than the total fraction of radiolabelled peptides involved in the alkali-sensitive complex B (Fig. 2). This suggests that the alkali-sensitive complex B is not entirely composed of phospho-triesters that can be cleaved by the enzymatic activity which may be due to a heterogeneity in size and composition of the peptide ligands involved in the complexes. Relatively large ligands are potentially not released by the enzyme and the substrate specificity could also be influenced by the structure of the hydroxy amino acid esterified with the internucleotide phosphate. It is well known that phospho-triesters are cleaved preferentially between the phosphate and phenolic ligands. For example, Parathion (diethyl-p-nitrophenyl mono thio phosphate) is cleaved by phosphotriesterases to result in nitro phenol [20]. Thus it is possible that the radiolabelled peptides released by the enzyme reflect phospho-triesters where the tyrosyl residue is involved while peptides esterified by other hydroxy amino groups are not cleaved by the enzyme.

Theoretically, phospho-triesters between hydroxy amino acids and internucleotide phosphates could also be cleaved by the hydrolase at the internucleotide-phosphate bonds which would result in non-migrating phosphodiesters. However, after digestion with the phosphotriesterase-containing culture broth the amount of phosphotriesterase-sensitive material in the residual fraction was not increased (not shown).

Interestingly, the purified protein considered to represent the enzymatic activity of the culture broth could not release radiolabelled peptides from the nucleotide(s)-peptide(s) fractions (not shown). From this it could be suggested that the release of the radiolabelled peptides by incubation with the culture broth is not due to the phosphotriesterase activity. However, the culture broth and the purified enzyme are known to exhibit different substrate-specificities. For example, Soman (1,2,2-trimethyl- propyl-methylphosphonofluoridate) is hydrolyzed by incubation with the culture broth while it is no substrate for the purified enzyme [23]. It is unknown whether the culture broth of S. lividans transformed with the plasmid pRYE1 contains different phosphotriesterases or whether unknown factors contained in the culture broth induce additional substrate specificities.

D. Energy-minimized computer models. Sterical probability is a prerequisite for the consideration of phospho-triesters between hydroxy amino acids in (poly)peptides and internucleotide phosphate groups in DNA. Using the coordinates from data bases, energy-minimized computer models were constructed composed of random peptides containing hydroxy amino acid residues esterified with the internucleotide phosphates of DNA. Fig. 8, displays the three-dimensional model of a tri-peptide comprising a tyrosine residue esterified in O4-position with an internucleotide phosphate in DNA. The models show that peptides of this type could well exist without sterical hindrance and distortion of DNA. Moreover, the models indicate that the existence of internucleotide polypeptide linkers, previously proposed on the basis of the well documented protease-inducible alkali lability of DNA [5, 6], are unlikely because of insufficient space between two adjacent nucleotides. It is obvious that the integration of even a small peptide between two adjacent nucleotides would distort the double helix tremendously.

**DISCUSSION**

The general procedure designed previously to identify and to analyse linking-groups between peptides and nucleotides in alkali-denatured DNA [7] has been found to be appropriate to identify additional linking-groups existing in native DNA that are not stable in alkali and, consequently, not detectable in DNA isolated by prolonged alkaline cell lysis.

One class of peptides that is not detectable in alkali-denatured DNA consists of phospho-peptides that are tightly associated with native DNA, however, not covalently bound to DNA. Most likely, this fraction of peptides is related with that released from native DNA by means of slightly alkaline (pH 9.5) buffers exhibiting inhibition of *in vitro* transcription [3].

A second class of peptides is released from nucleotides by strong alkali. The characteristics of this alkali-inducible release and the fact that increased levels of peptide-nucleotide phosphodiesters could be detected after the alkali treatment implies a structure of covalent nature. Since alkali cannot be considered to catalyze the formation of phosphodiester bonds between hydroxy amino acids and nucleotide phosphates [16-18] it is conceivable to conclude that the increased levels of phosphodiesters found after alkali treatment reflect products of a hydrolytic cleavage. Consequently, phospho-triesters between hydroxy amino acid residues in peptides and internucleotide phosphate groups were considered as hypothetical structures which could result in the cleavage products detected. The existence of such structures is indirectly supported by the reaction products formed after alkali treatment of the chemically prepared model compound composed essentially of L-tyrosine esterified with the internucleotide phosphate of deoxythymidyl-(3'→5') deoxy thymidine [unpublished]. Further support for the existence
of phospho-triester structures in the alkali-sensitive complex is based on its (partial) cleavage by a culture broth of S. lividans (pRYEl) exhibiting Parathion and Soman-hydrolyzing phosphotriesterase activities [15, 22]. Direct evidence for the existence of peptide-nucleotide phospho-triesters is based on the reaction products formed during base-specific chemical cleavage reactions. Thus, since there is experimental evidence for phosphotriesters in the complex B and since phospho-triesters are rather stable under physiological conditions and sterically compatible with the DNA structure we conclude that the bonds between peptides and nucleotides in the alkali-sensitive complex B are best explained by phospho-triester linkages.

(Poly)peptide-DNA phospho-triesters and their (partial) hydrolytic conversion into phosphodiesterphosphates, signifying strand scissions on the DNA level, could also well explain the protease-inducible alkali-lability of DNA that has been previously interpreted as evidence for protein linkers in DNA [5, 6]. In the light of the new results the well documented change in size of denatured DNA observed after prolonged protease treatment [5, 6] could as well be explained by the triester structures. In the native (poly)peptide-DNA complexes the phospho-triester linkage might be rather alkali-stable because protected or otherwise stabilized by the protein(s) while prolonged digestion with proteases is likely to render the phospho-triester linkages more alkali-sensitive resulting in an increased number of strand breaks.

In order to characterize the linking-groups between protein and DNA it was essential to digest DNA preparations with a protease and with nucleases. Consequently, the results shown in this paper give no information about the size of the proteinaceous material involved in the native complexes. However, it is highly likely that the linkage-groups characterized in this paper reflect the binding sites of polypeptides that have been detected in highly purified DNA preparations from different organisms and by different techniques including (1) digestion of milligram-amounts of highly purified DNA by nucleases followed by examination of the polypeptides by SDS / polyacrylamide gel electrophoresis and Coomassie blue staining [4], (2) radioiodination of DNA preparations followed by degradation of DNA and analysis of the residual radiolabelled polypeptides by combined SDS / polyacrylamide gel electrophoresis and autoradiography [24], and (3) radiolabelling of polypeptides associated with DNA by nick-translation in presence of α-[32P]dCTP and analysis of the polypeptides and their associated residual radiolabelled nucleotides by combined SDS / polyacrylamide gel electrophoresis and autoradiography [25]. DNA-polypeptide complexes of this kind were found in DNA at an average distance of ~10 kbp [26], however, there is evidence indicating that the distribution along DNA is non-random [27, 28]. Since the physico-chemical characteristics of the polypeptides involved in the complexes are different from those of topoisomerases it has been concluded that at least the majority of the covalent complexes do not reflect topoisomerase molecules transiently bound to DNA [29]. DNA fragments associated with the tightly-bound nuclear polypeptides are specifically retained on nitrocellulose filters [26] and the DNA sequences contained in the retained fraction represent a sub-set of DNA sequences [27, 28] highly overlapping with the residual DNA sequences retained in nuclear matrix preparations [30] which points to the physiological significance of the complexes. The sequence-dependent location of those polypeptides in DNA [27, 28, 30] is consistent with the results indicating that the linking-groups characterized in this paper show preferences with respect to their locations.

REFERENCES