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CROSS-LINKING OF PROTEINS TO DNA IN NEWLY SYNTHESIZED CHROMATIN BY DIISOPROPYLFLUOROPHOSPHATE, A SERINE PROTEASE INHIBITOR

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SUMMARY When nascent DNA of SV40 pulse labeled with [α-^{32}P]dCTP in a permeable cell system was treated in situ with diisopropylfluorophosphate (DFP), a significant fraction of radioactivity was found to be covalently complexed with proteins. The adduct formation was demonstrated by density separation in CsCl, selective precipitation of the complexed DNA with SDS-KCl, and visualization of cross-linked protiens after SDS-PAGE. No cross-linking occurred with mature SV40 chromatin labeled in vivo and extracted from nuclei of infected cells. The DFP-induced DNA-protein cross-linking reaction appears to involve the protein's sulfhydryl groups since pretreatment with some sulfhydryl reagents completely inhibited the reaction.

Protein-nucleic acid cross-linking techniques have been successfully used to obtain direct evidence for the presence of specific interaction between these macromolecules. Recently we employed an UV-induced DNA-protein cross-linking reaction to identify proteins interacting with newly replicated DNA in SV40-infected cells (1). Nascent SV40 DNA in infected cells permeabilized with saponin was labeled with [α-^{32}P]dCTP and BrdUTP. The labeled cells were then irradiated with UV light (254 nm) and were treated extensively with DNase I. Proteins with radioactive DNA tags were separated by SDS-PAGE and visualized by autoradiography. In the course of this study it was noted that a significant level of background cross-linking occurred without UV treatment whenever DFP was included as a protease inhibitor in the reaction mixture.

In this report we present evidence that DFP actually induces covalent linkages between proteins and DNA newly synthesized in vitro.

MATERIALS AND METHODS

Labeling of nascent SV40 DNA in permeable cells The procedure has been described in detail previously (1). Briefly, CV-1 cells (1.5×10^6 cells per reaction) were permeabilized with 0.05 % saponin and incubated at 37°C with 50 μl of the reaction mixture containing 50 mM Tris-HCl (pH 7.4), 120 mM

Abbreviations used: DFP, diisopropylfluorophosphate; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; SLS, sodium N-lauroyl sarcocinate; TPCK, L-1-tosylamide-2-phenylethyl chloromethy ketone; PAGE, polyacrylamide gel electrophoresis.

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potassium acetate, 5 mM $MgCl_2$, 2 mM ATP, 0.1 mM dATP, dGTP and dTTP, 1 μM [α-^{32}P]dCTP (100-200 Ci/mmol), and 10 μg/ml each of chymostatin and leupeptin. After a short pulse, the reaction was terminated by the addition of 20 μg/ml aphidicolin followed by rapid cooling to 0°C. The cells were treated with 2 mM DFP for 2 min at 37°C, either during the DNA synthesis reaction or immediately after the pulse in the presence of 0.1 mM unlabeled dCTP. DFP was prepared as a 0.2 M solution in isopropanol and stored at -20°C.

CsCl density gradient centrifugation The labeled cells were pelleted by centrifugation and resuspended in 40 μl of 80 mM NaCl, 5 mM $MgCl_2$, 0.15 M sucrose, 50 mM Tris-HCl (pH 7.4), 1 mM PMSF, and 50 μg/ml each of chymostatin and leupeptin. The suspension was treated with 5 μg/ml DNase I (Boehringer Mannheim, grade I) for 30 min at 15°C to shear the chromatin DNA. The incorporated radioactivity remained acid-insoluble after this treatment. The cells were then lysed with 0.2 ml of 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 3 M NaCl, and 0.5 % SLS (2), centrifuged at 10,000 rpm to remove aggregates, and mixed with saturated CsCl solution to make the final density of 1.700 as determined refractometrically. The mixture was transferred to 5-ml polypropylene centrifuge tubes, overlayed with distilled water, and centrifuged for 32 h at 45,000 rpm (20°C) in a Hitachi swing rotor RPS 65TA. Gradients were fractionated from the bottom into 31 fractions. Aliquots were removed from each fraction to determine the density on a refractometer, and were spotted onto Whatman GF/C filters for scintillation counting with toluene-based scintillation fluid. The filters were washed with 1 N HCl and 1% sodium pyrophosphate with several changes before drying.

SDS-K^+ precipitation method After shearing the DNA by the low concentration DNase treatment, the labeled cells were lysed with 1 ml of 10 mM Tris-HCl (pH 7.4), 10 mM EDTA and 1 % SDS, and centrifuged at 10,000 rpm. To 0.5 ml of the lysate placed in a 1.5 ml Eppendorf tube, 64 μl of 2 M KCl was added and the mixture incubated on ice for 10 min after vortexing. The white precipitate formed was pelleted by centrifugation at 4,500 rpm for 4 min (4°C) and washed 3 times with 1 ml of ice cold 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 M KCl by resuspending and centrifuging each time. The washed precipitate was dissolved in 1 N HCl, neutralized with the same volume of 1 N NaOH and applied onto glass fiber filters for ^{32}P counting.

In vivo labeling and extraction of SV40 chromatin Confluent CV-1 cells (6 dishes) were infected with SV40 at a multiplicity of 50 per cell and harvested after 37 h. SV40 chromatin replicating in the cell was bulk-labeled with 5 μCi/ml [^{3}H]thymidine (45 Ci/mmol) during the last 16 h in Eagle's MEM supplemented with 3 % bovine serum. The cells were scraped off the dishes into a hypotonic buffer containing 10 mM HEPES (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$ and 0.5 mM DTT (3), disrupted by passing through an 18 gauge needle, and then spun at 3,000 x g for 5 min. To the pelleted nuclei was added 0.4 ml of the hypotonic buffer, and the suspension was incubated on ice for 3 h with occasional agitation. The nuclear extract containing about 50% of the total SV40 chromatin was recovered as a supernatant after centrifugation at 8,000 x g for 10 min. The nuclear extract (30 μl) was combined with the reaction mixture for permeable cells lacking [α-^{32}P]dCTP and incubated at 37°C for 2 min in the presence or absence of 2 mM DFP. After destroying noncovalent associations with the lysis buffer containing SLS and NaCl, the labeled SV40 chromatin was subjected to CsCl density gradient centrifugation as described above for permeable cells.

Gel electrophoresis of cross-linked proteins After treatment with DFP under various conditions, the ^{32}P-labeled nascent SV40 DNA in permeabilized cells was digested extensively with 500 μg/ml DNase I for 30 min at 37°C. This step leaves only a fractional amount of labeled DNA covalently attached to proteins, marking the cross-linked proteins as radioactive tags. The tagged proteins were separated by SDS-PAGE (14 % acrylamide) and visualized by autoradiography as described previously (1).

RESULTS

It has been demonstrated that the permeable cell system used in the present study allows one to pursue the replicational process of SV40 DNA in situ (4). More than 70% of the nucleotide incorporation into DNA was accounted for by the replicative synthesis of SV40 DNA. Under the standard conditions

shown in Materials and Methods the incorporation continues for more than 5 min. In the presence of 2 mM DFP, the incorporation started to fall off after 1 min and ceased to increase as early as 2 min after the start of incorporation. However, the very initial rate of DNA synthesis was not affected by DFP (results not shown). It appears, therefore, that DFP does not inhibit the DNA polymerization reaction per se but interferes with subsequent processes of chain elongation.

Formation of covalent linkage between proteins and nascent DNA synthesized in the presence of DFP was clearly shown by density gradient separation in CsCl (Fig. 1). This is a sensitive method to detect DNA-protein adducts and has been utilized extensively (2, 5, 6). When the permeable cells were treated with DFP for 2 min, about 40% of the labeled DNA was found in the low density region (ρ<1.50) as expected for DNA-protein covalent complexes (Fig. 1B). In the absence of DFP, less than 10% was recovered in this region, and most of the DNA had the normal density of SV40 DNA (ρ=1.70) (Fig. 1A).

This result was confirmed by the other method called SDS-K^+ precipitation which has been used to demonstrate the covalent complexes of topoisomerases

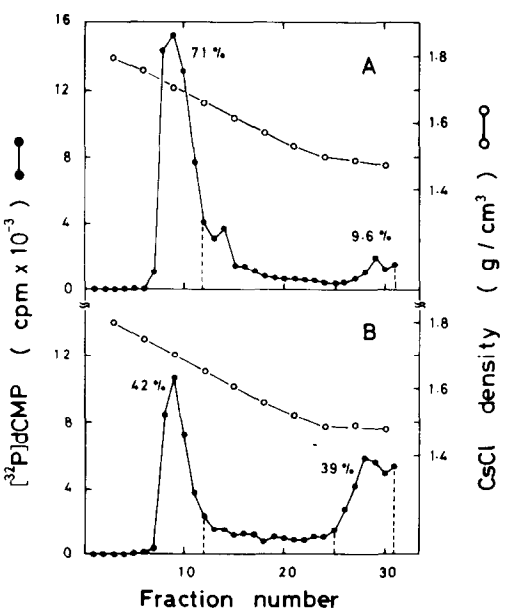


Fig. 1 (left panel) CsCl density gradient centrifugation of pulse labeled SV40 DNA. See Materials and Methods for experimental details. Permeabilized cells were pulsed for 2 min and incubated with (B) or without (A) 2 mM DFP before centrifugation. In the figure are indicated percentages of radioactivity recovered in the normal density region (fraction No. 7-12) and in the low density region (No. 25-31).

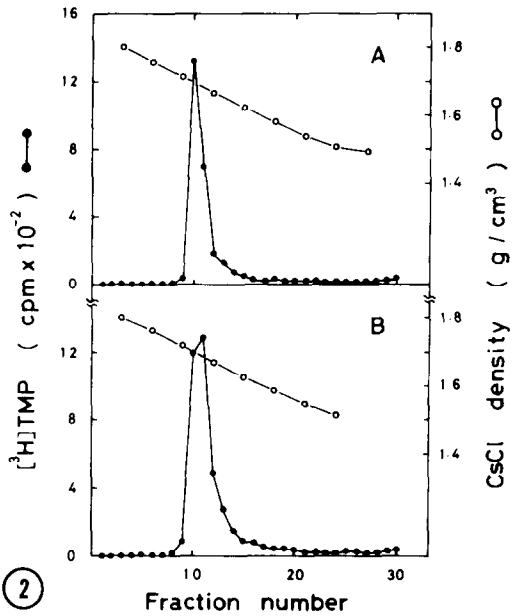
Table 1 Formation of DFP-induced DNA-protein adducts as revealed by SDS-K^+ precipitation

Treatment	% cpm precipitated
None	7.0
2 mM DFP, during pulse	31
2 mM DFP, after pulse	29
2 mM DFP plus 10 mM NEM, after pulse	2.6

with DNA formed as an enzyme-substrate intermediate (7, 8). The procedure involves the formation of SDS-protein complexes followed by precipitation of the complexes with KCl which also precipitates the labeled DNA covalently attached to proteins while leaving protein-free DNA in solution. As shown in Table 1, the proportion of DNA precipitated from the SDS extract of DFP-treated cells was comparable to that which floated on the CsCl gradient, indicating again that DFP acts as a DNA-protein cross-linker in nascent chromatin. When the cells were treated with DFP after terminating DNA synthesis with aphidicolin, a similar level of cross-linking occurred. This result implys that DFP acts somewhere after the polymerization step, and also eliminates the possibility that DFP is facilitating a direct modification of proteins with unpolymerized [α-^{32}P]dCTP which is diluted out at this point by excess unlabeled dCTP.

The cross-linking effect of DFP has not been observed in other systems despite the rather common usage of this agent. In fact, DFP is sometimes included in media for preparation of chromatin to suppress proteolytic degradation (9). It is likely, therefore, that chromatin in general is not extensively cross-linked by DFP, but rather the effect is unique to newly replicated chromatin. To test this possibility, SV40 chromatin was extracted from the nuclei of infected cells, treated with DFP, and analysed with CsCl gradient centrifugation. Under the labeling protocol used here, the ^{3}H label incorporated into DNA represents the nonreplicating chromatin in mature configuration. The absence, shown in Fig. 2B, of the DFP-induced density shift observed in Fig. 1B supports the above assumption.

More direct evidence for the cross-linking was obtained by the gel electrophoretic procedure utilized for identifying cross-linked proteins in UV-



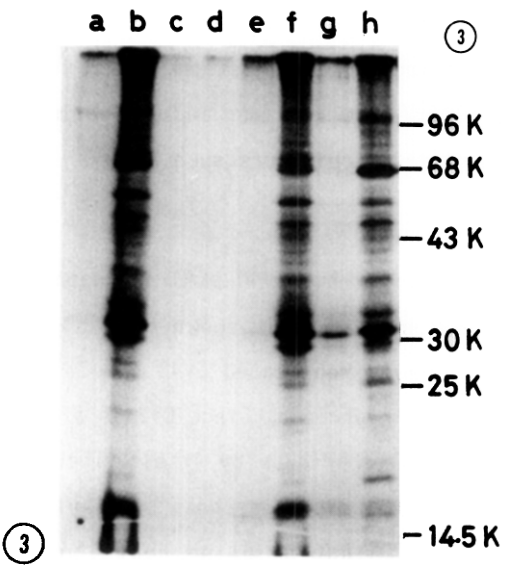


Fig. 2 (right panel) CsCl density gradient centrifugation of mature SV40 chromatin. SV40 DNA in infected cells was labeled with [^{3}H]thymidine for 16 h, the minichromosome was extracted from the nuclei, and incubated with (B) or without (A) 2 mM DFP before centrifugation.

Fig. 3 Visualization of cross-linked proteins by SDS-PAGE. The permeable cells were pulsed for 2 min and treated for 2 min at 37°C with the following agents. (a) no agent, (b) 2 mM DFP, (c) 10 mM NEM then 2 mM DFP, (d) 10 mM PCMB then 2 mM DFP, (e) 2 mM DFP with 10 mM NEM, (f) 2 mM DFP then 10 mM PCMB, (g) extensive digestion with DNase I followed by 2 mM DFP, and (h) 2 mM DFP during the pulse. Molecular weights of marker proteins are indicated along the right margin.

irradiated chromatin (1). After the 2-min pulse labeling, the permeable cells were treated with DFP and other agents. The final autoradiogram is shown in Fig. 3. The occurrence of protein-DNA cross-linking was clearly dependent on the DFP treatment (lanes a and b). Some of the protein bands visualized here appear to correspond to those generated by UV cross-linking (1). A similar pattern was observed when DFP was present during the DNA synthesis (lane h). However, the cross-linking was almost abolished if DFP was preceded by the DNase digestion (lane g), suggesting that the polymeric nature of chromatin DNA or the higher structure of chromatin is necessary for the cross-linking to occur. Interestingly, the effect of DFP was completely canceled in the presence of NEM, an SH blocking agent (lane e). The same result was obtained by the SDS-K^+ precipitation procedure (Table 1). This agent does not seem to directly inactivate DFP since the inhibition was also observed when cells were pretreated with NEM, and DFP was added after a brief washing (lane c). This

observation suggests the involvement of the protein's sulfhydryl groups in the cross-linking reaction. PCMB, another SH blocker, also suppressed the cross-linking (lane d), but the cross-links once formed by DFP were not reversed by PCMB (lane f). The adduct formation was not observed with other serine protease inhibitors such as TPCK, PMSF, leupeptin, and chymostatin.

DISCUSSION

Using the SV40 DNA replication system in nucleotide-permeable cells, we have unequivocally shown that DFP induces the formation of protein-DNA adducts in newly replicated SV40 chromatin. The mechanism of cross-linking, however, is still unclear. Since DFP is a monofunctional agent, it can not form intermolecular bridges by itself. Therefore, it is likely that DFP first activates an amino acid side chain by alkylphosphorylation, then the adduct reacts with DNA eliminating diisopropylphosphate to form a stable cross-link between a protein and the DNA. The inhibitory effect of the sulfhydryl reagents on the cross-linking (Fig. 3) suggests that the thiol group of cysteine is the target of DFP. It is well established that DFP selectively inactivates some serine proteases by irreversibly modifying the hydroxyl group of a single serine residue at the active center. This serine OH is unique in that it has an extremely high nucleophilicity compared to other hydroxyl groups of the enzyme. Although there has been no evidence presented so far, it is possible to assume the existence of such unique thiol groups in proteins interacting with DNA when the similar nature of these chemical groups is taken into account.

The apparent specificity of the cross-linking reaction to newly replicated chromatin suggests that DFP recognizes some structural feature of replicating chromatin such as prenucleosomal DNA (10) or single stranded DNA (11) in the replication fork. Another intriguing interpretation for the failure to induce the DNA-protein adducts in mature SV40 chromatin extracted from nuclei (Fig. 2) is that the adduct formation mainly involves proteins associated with some nuclear structure, most likely the nuclear matrix. There is growing evidence implying that DNA replication complexes are attached to the nuclear matrix in eukaryotic cells (12), as well as in some DNA viruses including SV40 which replicate in nuclei (13, 14). Our preliminary experiments showed that most of the cross-linked proteins visualized by autoradiography (Fig. 3) were recovered in the residue fraction after treating the labeled nuclei with DNase

and a high salt buffer, suggesting that these proteins are constituents of the nuclear matrix.

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