Journal of Chromatography, 618 (1993) 277–288
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6817

Review

Electrophoretic and chromatographic separation methods used to reveal interstrand crosslinking of nucleic acids

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(First received December 18th, 1992; revised manuscript received March 3rd, 1993)

ABSTRACT

Several electrophoretic and chromatographic techniques, many of which have only been developed recently, provide sensitive methods for the detection and separation of DNA containing interstrand crosslinks such as those produced by many cancer chemotherapeutic drugs and photoactive psoralen derivatives. Most of the methods rely on the fact that the presence of such crosslinks prevent the complete denaturation of the two complimentary DNA strands by heat or alkali. A simple and highly sensitive neutral agarose gel electrophoresis method is particularly applicable to detailed time-course experiments of both total crosslink formation, and the "second-arm" of the crosslink reaction. This method separates denatured single-stranded from double-stranded DNA which has reannealed as a result of an interstrand crosslink. Polyacrylamide gel-based assays using denaturing gels are more suited to the separation of smaller crosslinked DNA fragments and, in particular, small oligonucleotides on high-percentage gels. In addition, they provide methods for the determination of the exact base position and sequence selectivity of crosslink formation. Sephadex chromatography and high-performance liquid chromatography can separate small crosslinked oligonucleotides from non-crosslinked duplexes, and the hydroxyapatite column chromatographic separation of single- and double-stranded cellular DNA can be used to quantitate the level of interstrand crosslinking present in the bulk of the genome. Finally, the analysis of damage by crosslinking agents, and its repair, at the level of specific genes can be achieved by hybridization with specific probes following membrane transfer from neutral agarose gels used to fractionate restricted and fully denatured genomic DNA from drug-treated cells.

CONTENTS

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0378-4347/93/$24.00

LIST OF ABBREVIATIONS

EDTA	Ethylenediaminetetraacetic acid
HPLC	High-performance liquid chromatography
Kbp	Kilo base pair
NMR	Nuclear magnetic resonance

1. INTRODUCTION

Many agents used in the treatment of cancer are bifunctional and are able to crosslink biological macromolecules [1,2]. DNA is generally believed to be the most important cellular target and in many cases, *e.g.* the bifunctional alkylating agents, the formation of interstrand crosslinks may be the most relevant cytotoxic lesion [2]. Various psoralen derivatives, some of which have been used to treat psoriasis and have been used in numerous studies as probes to study the structure of nucleic acids, also bind to DNA and, upon exposure to long-wavelength ultraviolet light, covalently bind pyrimidine bases to form monoadducts and interstrand crosslinks [3]. DNA interstrand crosslinks are formed by an initial covalent reaction of drug with one strand of the DNA to form a monoadduct, which can be converted to a crosslink by a second reaction, with the other DNA strand. Not all monoadducts are converted to crosslinks and the "second-arm" reaction is often slow compared to the initial monoadduct formation.

Several direct and indirect biophysical and biochemical methods exist for the measurement of DNA interstrand crosslinking. These include cesium chloride [4] and alkaline sucrose [5] gradient centrifugation, selective removal of single-stranded DNA by S1 nuclease [5], chemical analysis [6,7] and the increased fluorescence of ethidium bromide in double-stranded DNA [8,9]. These techniques are in many cases insensitive and time-consuming, may require large amounts of DNA and drug, and are generally not applicable to detailed time-course experiments. The technique of alkaline elution developed by Kohn and co-workers [10,11] has become the most important technique for measuring the formation and repair of genomic interstrand crosslinks in cells at pharmacologically relevant doses. Recently, however, several chromatographic and, in particular, electrophoretic techniques have provided sensitive methods for the detection and separation of crosslinked products. These methods have also provided detailed information not obtainable from other techniques, such as the DNA sequence selectivity of crosslink formation and the analysis of crosslink formation and repair in specific gene sequences in cells.

2. GENERAL PRINCIPLE OF THE METHODS

The principle on which many electrophoretic and chromatographic methods for the separation of DNA containing an interstrand crosslink is based on the fact that such a lesion can prevent the complete denaturation of the two DNA strands. In consequence, upon appropriate renaturation conditions crosslinked DNA strands regain the reversible bihelical property, while non-crosslinked strands remain permanently denatured and single-stranded. Denaturation can be achieved by the addition of alkali, *e.g.* sodium hydroxide to pH 12–12.5, and renaturation by neutralisation with hydrochloric acid. Alternatively denaturation by heat can be used followed by rapid cooling. The temperature required for complete denaturation will depend on the size and composition of the DNA, and the duration of heating is important to avoid introducing strand breaks into the DNA.

The choice of denaturation conditions may depend on the particular method employed, and also on the crosslinking agent under investigation. Some crosslinks may be labile to extended heat treatments necessitating the use of alkaline denaturation. Alternatively in some rare cases drugs may induce alkali-labile sites. The production of DNA single-strand breaks by a drug, in addition to interstrand crosslinks, may also result in an inaccurate estimate of crosslinking in some assays. What is clear for all the methods to be discussed is that DNA of the highest quality is essential to produce reliable and accurate measurements of crosslinking.

3. AGAROSE GEL-BASED METHODS

A simple agarose gel electrophoresis method for the determination of DNA interstrand crosslinks based on the principle outlined above has recently been described [12]. The method is illustrated schematically in Fig. 1. ^{32}P-End-labelled linear plasmid DNA of appropriate length, such as the full-length pBR322 DNA (4363 base pairs), is used. Following complete denaturation–renaturation of the DNA the single strands are separated from the double-stranded DNAs which contain an interstrand crosslink, on a neutral agarose gel. Visualisation of the results is by autoradiography of the dried gel (Fig. 2) and accurate quantitation can be achieved by densitometry (Fig. 3). The method requires only 10–50 ng of DNA per lane and is particularly applicable to detailed time-course experiments of both total crosslink formation (Figs. 2a and 3) and, following removal of free drug, the "second-arm" of the crosslink reaction (Fig. 3). It is sensitive enough to follow the formation of crosslinks by slow and inefficient crosslinking agents, such as the chemotherapeutic agent busulphan [13], which have not previously been measured by physical procedures.

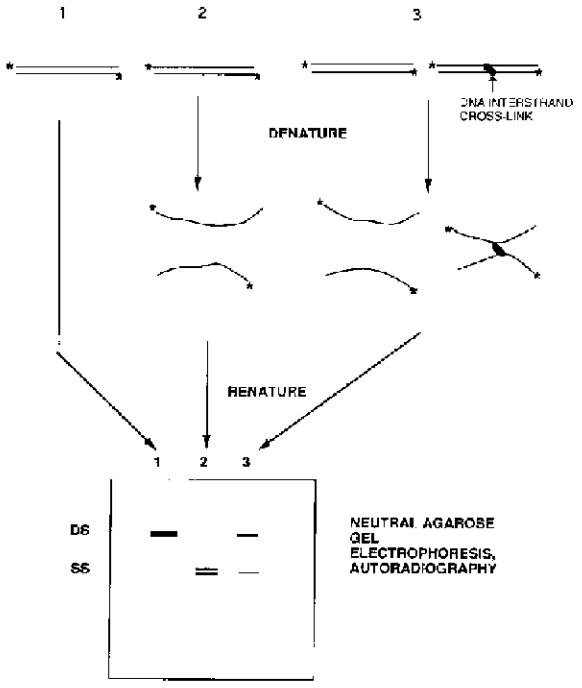

Fig. 1. Neutral agarose gel method to detect DNA interstrand crosslinking. Linear DNA end-labelled with ^{32}P (*) is used. In the control non-denatured sample (1) the DNA runs as double stranded (DS). In the control denatured sample (2) the DNA runs as single-stranded (SS). The presence of crosslinks in the drug-treated sample (3) prevents the DNA strands from denaturing completely which can reanneal and run as double-stranded in the neutral agarose gel.

Complete denaturation of the plasmid DNA can be achieved by either alkali or heat, but the most reproducible results are obtained using heat in an appropriate strand separation buffer such as 30% dimethylsulphoxide, 1 m*M* EDTA to prevent reancaling. This requires prior precipitation of the drug-treated DNA and choice of the buffer for the crosslinking reaction is therefore important. In particular, the use of buffers containing high salt or phosphate concentrations should be avoided because of their precipitation with ethanol and which do not subsequently dissolve easily in the strand separation buffer, making gel loading difficult. In strand separation buffer linear pBR322 DNA can be converted to >98% single-stranded by heating at 90°C for 2 min with no production of single-strand breakage. In addition no release of modified purines is observed under these conditions [12]. This can occur from alkylated DNA under conditions of elevated temperature such as boiling [14].

As an alternative to ^{32}P-labelling, non-labelled DNA can be used, but in this case up to 500 ng of DNA per lane is required, since quantitation by ethidium bromide staining of the gels is much less accurate. A correction is required in the calculation of the percent crosslinked DNA due to the lower efficiency of staining by ethidium of single-stranded DNA compared to double-stranded.

A limitation of the technique is that it is independent of the number of crosslinks per DNA molecule, the DNA being maintained as double-stranded if it contains either one or many crosslinks. It is therefore important when performing experiments to measure the time to reach maximum crosslinking that plateau levels are chosen below 100% of DNA molecules crosslinked since

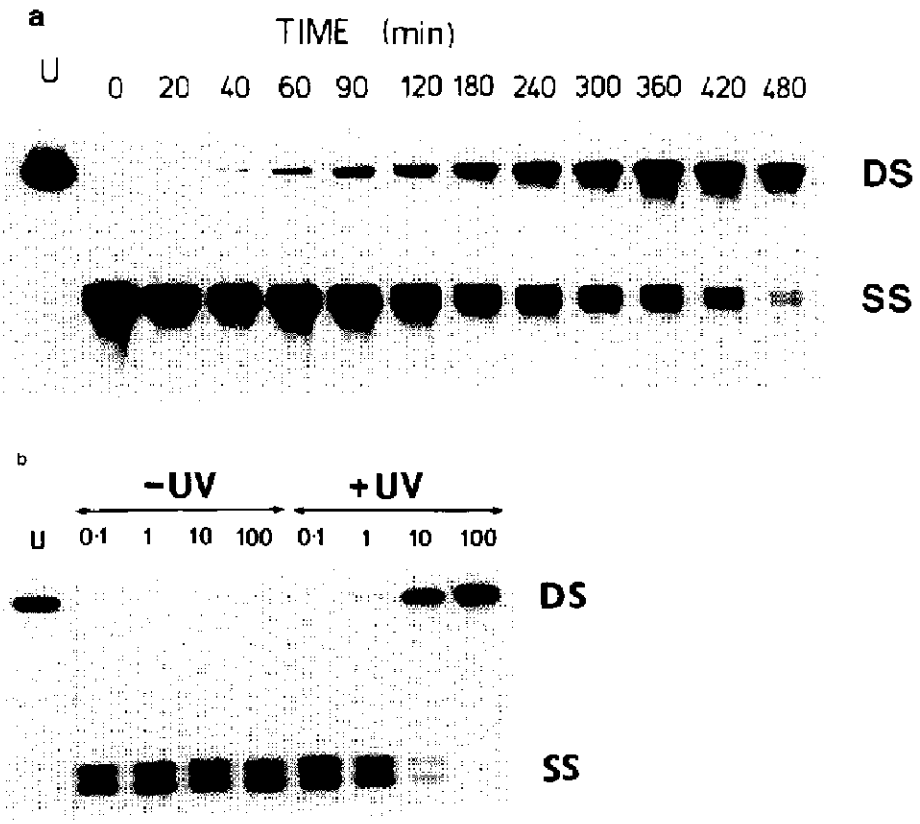

Fig. 2. (a) Autoradiograph of an agarose gel showing a time-course of crosslink formation for melphalan. ^{32}P-End-labelled DNA (4360 base pairs) was treated with drug (10 μM, 37°C) for the times shown, reaction stopped, DNA precipitated, dried, redissolved in strand separation buffer and denatured by heating for 2 min at 90°C and then immediately placed in an ice–water bath prior to loading on a 0.8% agarose gel. The gel was run for 16 h at 40 V, dried and autoradiographed. Bands correspond to DS and single stranded (SS) DNA. U is untreated, non-denatured DNA. (b) Autoradiograph of an agarose gel showing photo induced crosslinking by 8-methoxypsoralen. DNA was treated for 1 h at the doses shown (in μM), and then illuminated with UV where indicated prior to processing as described in (a).

the rate to achieve maximum crosslinking can only be determined under these conditions.

Other techniques utilising agarose gels have focused on the ability of interstrand crosslinks to alter the mobility of restriction fragments of plasmid DNAs. For example, it is reported that the electrophoretic mobility of a mixture of Hae III $\phi X174$ DNA fragments is reduced upon reaction with 4,5′,8-trimethylpsoralen and UV light in alkaline (pH 12.4) agarose gels without prior denaturation of the DNA [15]. Somewhat surprisingly, however, the shift in mobility for low concentrations of the psoralen was much greater than for high concentrations. The method required relatively large amounts of DNA per sample due to detection by ethidium bromide staining, and the alkaline agarose gels require continuous circulation of the buffer.

4. POLYACRYLAMIDE GEL-BASED METHODS

The separation of crosslinked DNA fragments can also be achieved using denaturing polyacrylamide sequencing gels following denaturation of the DNA. The presence of a high concentration of urea and an elevated running temperature during electrophoresis ensure complete separation of the DNA strands. Under these conditions cross-

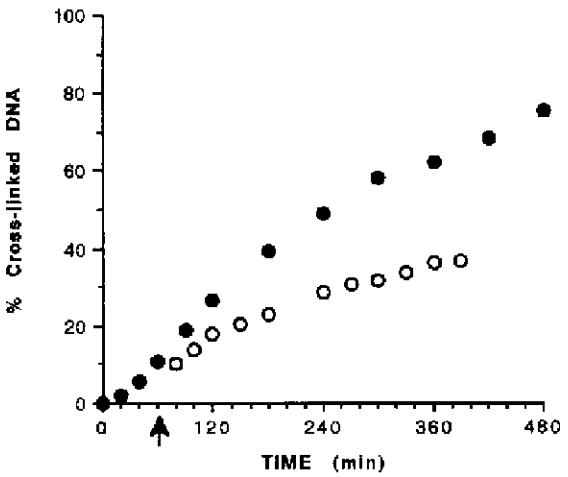

Fig. 3. Time-course of total and "second-arm" crosslink reaction for melphalan. Total crosslink formation (●) was determined by densitometry from the autoradiograph in Fig. 2a. The kinetics of the second-arm reaction (○) were determined by stopping the reaction at the time indicated (arrow) after allowing approximately 10% crosslinking to occur. Following precipitation the DNA was resuspended in drug-free buffer and incubated for the times shown prior to denaturation and gel separation.

linked molecules are again retarded in the gel. The size of DNA fragments used for this assay differs from that employed in the agarose-based methods, the polyacrylamide gels being suited to small fragments and, in particular, to the separation of small oligonucleotides on high-percentage (up to 20%) gels. Bands from heavily loaded samples can be visualised by UV shadowing but prior end-labeling of the DNA with ^{32}P affords a high degree of sensitivity for the detection of crosslinked products which often constitute only a small percentage of total reaction products. This assay is less suitable for detailed time-course experiments than the simpler agarose gel-based method, but the use of synthetic oligonucleotides of defined sequence makes it a powerful technique for examining the sequence selectivity of crosslink formation.

4.1. Sequence selectivity of crosslink formation

Using end-labelled duplex oligonucleotides, each synthesised to contain only a single potentially crosslinkable site, it is possible to infer the relative crosslinking preferences of a particular drug by comparing the fraction of the crosslinked products retarded in the gel. This has been shown elegantly for 4,5′,8-trimethylpsoralen crosslinking in a series of synthesized fragments containing all possible pyrimidine and purine base pair combinations and revealed that the crosslinking appeared to be dependent not only on the dinucleotide sequence (with preferred crosslinking at 5′-TA sites), but also on the bases flanking the dinucleotide, and in some cases the long-range sequence context of the DNA [16]. Similarly this method also demonstrated the preference for 5′-GC sequences, and the influence of neighbouring bases, for crosslinking by the antitumour agent mitomycin C [17].

In more complex oligonucleotides which contain several potentially crosslinkable sites it is possible to obtain several slow-migrating crosslinked products of differing mobilities dependent on the position of the crosslink in the duplex [18] (Fig. 4), with products containing crosslinks towards the middle of the duplex tending to be retarded more in the gels than those in which the crosslink is near or at the end. The relative band intensities rank the order of preference of the crosslinking sites but this method alone cannot give an exact assignment of the base sites involved in the crosslinking. This can, however, be achieved using recently developed methods.

One such method is outlined schematically in Fig. 5 and utilises the fact that for agents such as the nitrogen mustards, which produce alkylations primarily at the guanine N7 position, the alkylation can be quantitatively converted into a strand break by treatment with hot piperidine [19]. Therefore, if duplex oligonucleotides are used which are end-labelled only on one strand, isolation of the crosslinked product followed by cleavage with piperidine can reveal the exact guanine base involved in the crosslink on the labelled strand on a second denaturing sequencing gel of single-base resolution. If the procedure is then repeated with the other strand of the oligonucleotide labelled, the other guanine involved in the interstrand crosslink can be pinpointed. The procedure is illustrated for two of the crosslinked

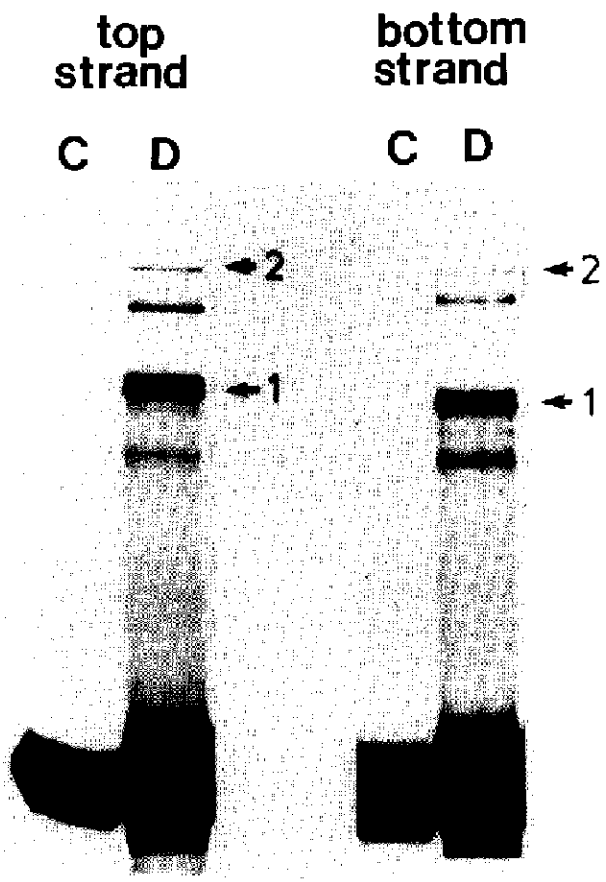

Fig. 4. Autoradiograph of a denaturing 20% polyacrylamide gel showing the crosslinked products of the reaction of a double-stranded oligonucleotide 23-mer (see Fig. 6), singly end-labelled either on the top or bottom strand, and reacted with the cross linking agent 2,5-diaziridinyl-1,4-benzoquinone. C and D are control and drug-treated samples, respectively. The products indicated were excised from the gel for further analysis (see Fig. 6). For further experimental details see ref. 21.

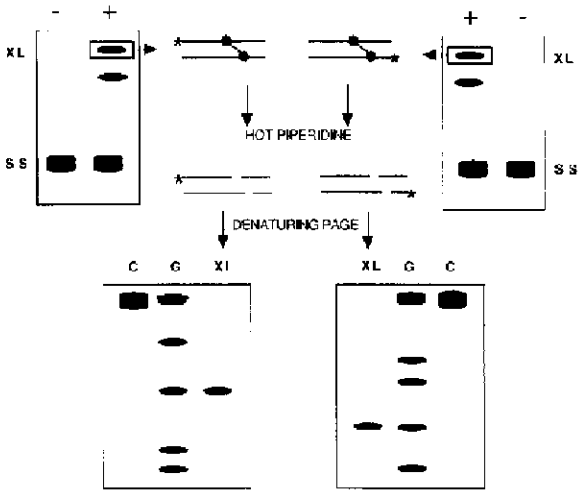

Fig. 5. Determination of the sequence selectivity of crosslink formation using the piperidine cleavage method. Crosslinked products excised from an initial denaturing polyacrylamide gel such as that shown in Fig. 4, using oligonucleotides singly end labelled (*) on the top or bottom strand, are subjected to cleavage at sites of guanine-N7 alkylation by piperidine plus heat. Products are separated on a second denaturing polyacrylamide gel to reveal the site of cleavage. XL is the crosslinked product, C control non-cleaved oligonucleotide and G a marker guanine lane to allow exact assignment.

products shown in Fig. 4, band 1 being the major product and band 2 a minor product. Fig. 6 shows the resulting second polyacrylamide sequencing gel for these two bands where either the top or bottom strands are labelled. Following the isolation and quantitative cleavage at guanine-N7 alkylation sites of the purified bands with hot piperidine, the crosslink sites can be assigned to a 5′-GAC sequence for the major product 1 and at a 5′-GC site for the minor product 2. This method was first utilised by Millard *et al.* [20] to show the preferential (and unexpected) site of preferential crosslinking by the nitrogen mustard mechlorethamine to be across three base pairs in a 5′-GNC sequence, and has now more recently been used to reveal preferential crosslinking sites for other groups of antitumour alkylating agents such as the bioreductive aziridinylbenzoquinones [21]. The conditions employed should be such that drug single-hit kinetics are used since crosslinked products which also contain monoalkylations at other guanine-N7 sites make the subsequent cleavage patterns difficult, and sometimes impossible, to interpret.

The above assay is necessarily limited to those agents which produce piperidine/heat labile crosslinking through guanine-N7 sites. Another assay developed by Weidner *et al.* [22] can be used for non-piperidine cleaved crosslinks. In this method singly end-labelled, singly crosslinked DNA fragments are again isolated by denaturing polyacrylamide gel electrophoresis and purified. These are then subjected to single-hit random cleavage using iron(II)-EDTA [23] and the fragments again separated by single nucleotide–reso-

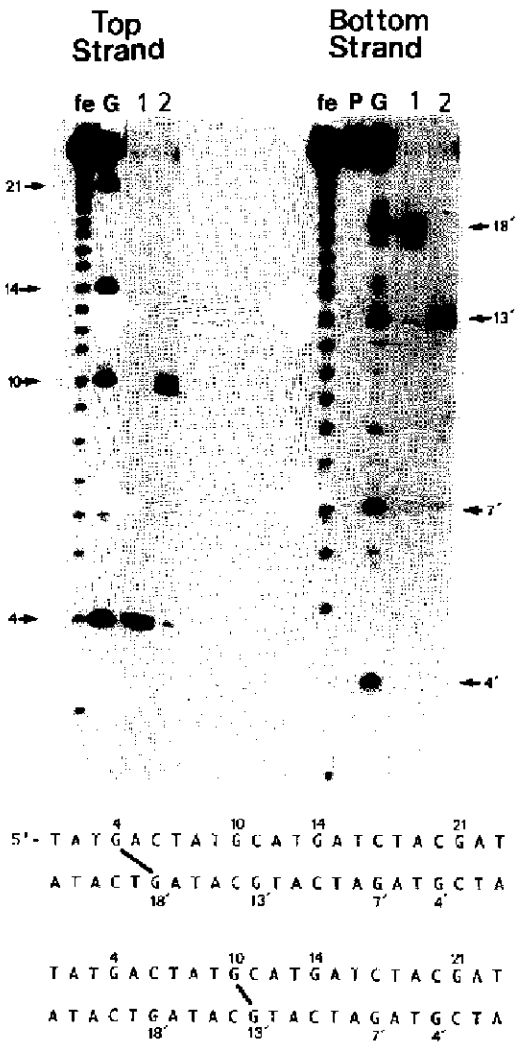

Fig. 6. Assignment of the sites of crosslinking for the major band 1 and the minor product band 2 excised from the denaturing polyacrylamide gel shown in Fig. 4. Purified products (1 and 2) were cleaved with hot piperidine and run on a second gel. Assignment of the crosslinking site was achieved by comparison with a guanine-specific lane (G), the numbering of the base positions shown by arrows (′ indicates bottom strand). The sequence of the oligonucleotide is shown below the gel with the guanine positions numbered. Also shown on the gels is a sample resulting from cleavage of the oligonucleotide with Fe–EDTA (fe) showing the position of every base, and a control lane, non drug-treated, but treated with piperidine (P). The results indicate the assignment of the crosslinking sites to a 5′-GAC sequence between bases 4 and 18′ for band 1, and at a 5′-GC site between bases 10 and 13′ for band 2, which are shown diagramatically below the gel.

lution denaturing polyacrylamide gel electrophoresis. Under such conditions random cleavage to the radiolabelled side of the crosslink in the DNA provides short labelled fragments differing in size by one nucleotide. Cleavage at any other site, on either strand, should afford much longer radiolabelled fragments. The electrophoretic separation of the fragment mixture produces a discontinuity in the gel diagnostic for the crosslink nucleotide site on the labelled strand, and the procedure can then be repeated for the other strand. The method is technically demanding and requires relatively large amounts of highly labelled oligonucleotide, particularly because the iron(II)–EDTA cleavage reaction is often inefficient. Nevertheless this is a useful technique which has revealed important information on the preferential sites of crosslinking by, *e.g.*, psoralen [22], mitomycin C [24] and cisplatin [25], largely confirming data obtained by other methods.

Other methods employing denaturing polyacrylamide sequencing gels for the detection of sites of DNA interstrand crosslinking rely on the fact that a crosslink blocks the production of various enzymes. For example, a double-stranded circular DNA having a single nick at a specific site was reacted with 4′-hydroxymethyl-4,5′-8-trimethylpsoralen plus light and used as a substrate for nick-translation with *Escherichia coli* DNA polymerase I [26]. The termination sites revealed on the sequencing gel suggested that the enzyme will usually copy past psoralen monoadducts but was blocked by a crosslink. In assays of this type, however, it is difficult to assess the true specificity for interstrand crosslinking compared with monoadducts and intrastrand adducts.

5. CHROMATOGRAPHIC METHODS

5.1. Hydroxyapatite column chromatography

Single- and double-stranded cellular DNA can be separated using hydroxyapatite (*e.g.* DNA-grade Bio-Gel HTP from Bio-Rad Labs., Hemel Hempstead, UK). High-molecular-mass DNA must be radiolabelled by prior incubation of cells with [^{3}H]- or [^{14}C]thymidine, followed by a chase period in label-free medium. DNA is extracted

from drug-treated cells, purified by standard procedures and denatured and renatured by either the alkali–acid or heating–cooling treatments. If heating is chosen 100°C is generally required for complete denaturation of the genomic DNA, but the time of heating is critical. A period of 3 min appears adequate and times greater than 5 min are not recommended as this can produce strand breaks in the DNA [27].

Typically 10 μg of DNA are then loaded onto a 1-ml packed volume of hydroxyapatite equilibrated with phosphate buffer (0.1–0.5 *M*), pH 6.8 [5]. Alternatively, the hydroxyapatite can be pipetted directly into the DNA sample and the solution loaded into a syringe barrel containing a pad of pyrex wool [28]. Elution is performed using a linear gradient of 0.05–0.4 *M* phosphate pumped through at 0.5–1 ml min^{-1} [5,28]. Fractions can then be counted directly by scintillation counting or as trichloroacetic acid insoluble radioactivity. The whole procedure can be successfully carried out at room temperature [5], but eluting the columns at 60°C can improve the recovery of DNA which is typically around 90% [28].

Permanently denatured, single-stranded DNA elutes first, followed by the crosslinked, reversible renatured DNA. The percentage of DNA that reversibly renatures in control samples is typically 5–10% and is related to the average molecular mass of the DNA sample. When calculating crosslink yields it is important to know the approximate molecular mass of the DNA because the sensitivity of the crosslink yield measurement is proportional to the size of the DNA molecules. Extreme care should therefore be taken in the isolation and denaturation of the DNA to avoid excessive shearing. The molecular mass can be estimated following the denaturation–renaturation step, but before the hydroxyapatite chromatography, by sedimentation in neutral or alkaline sucrose gradients. This is, however, not straightforward as the sedimentation profile of denatured crosslinked DNA is complex because of the mixture of crosslinked and permanently denatured strands [29]. The combined method is, however, relatively sensitive and can detect psoralen crosslinks at the level of about 1–3 per *E. coli* genome [$2.5 \cdot 10^9$ relative molecular mass (M_r)]. An alternative and less time-consuming approach involves fragmentation of purified double-stranded DNA by vortex-shearing to a unit length of $1 \cdot 10^7$ M_r [5] although the sensitivity of the method is necessarily lowered.

5.2. Sephadex chromatography

Crosslinked oligonucleotides can be separated from non-crosslinked duplexes using Sephadex chromatography. Sephadex G-25 (superfine, Pharmacia LKB, Uppsala, Sweden) is suitable for the separation of small oligonucleotides up to decamers, and Sephadex G-50 (DNA grade) gel for decamers and higher oligomers with 0.02 *M* ammonium bicarbonate as eluent [30]. Typically, columns 56 cm × 5 cm I.D. in size can be loaded with 10–15 μmol of mononucleotide units. The crosslinked oligonucleotide complex elutes in the void volume before the parent strands.

5.3. High-performance liquid chromatography

Crosslinked oligonucleotides can also be separated successfully using high-performance liquid chromatography (HPLC). For example, reversed-phase Beckman RPSC, C_3 Ultrapore columns (Beckman, Irvine, CA, USA) have been used to separate mitomycin C crosslinked oligonucleotides using 0.1 *M* triethylammonium acetate buffer pH 7 and acetonitrile as eluent, either isocratically or in a linear concentration gradient [30] (Fig. 7). When large-scale separations and purifications are required a crude initial separation using Sephadex chromatography is advisable (see above) to remove most of the unmodified duplexes and excess drug products.

Covalently crosslinked base products have been separated by HPLC from DNA treated with chemotherapeutic alkylating agents and then hydrolysed to the individual bases [7,31]. Such analysis, however, does not distinguish between products formed as a result of inter- or intrastrand crosslinks.

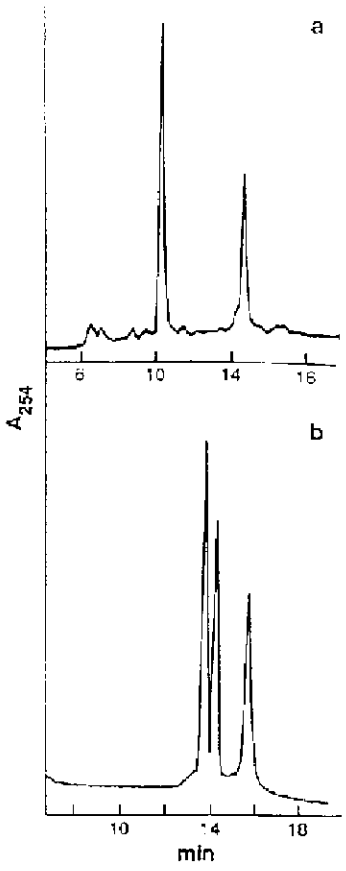

Fig. 7. HPLC of oligonucleotides crosslinked with mitomycin C using reversed-phase C_3 Ultrapore (Beckman) columns. (a) The duplex d(TACGTA) runs at 10.2 min in the absence of a crosslink and at 14.3 min when crosslinked by mitomycin C between its two guanine residues. Gradient, 5.7–13.8% acetonitrile in 0.1 *M* triethylamine, pH 7, in 36 min; flow rate, 1 ml/min. (b) Complementary oligonucleotides d[TATATCGTATAT] (13.4 min) and d[ATATACGATATA] (14 min), and their crosslinked duplex (15.8 min). Gradient, 6–18% acetonitrile in 0.1 *M* triethylamine, pH 7; flow-rate 1.0 ml/min. (Figure taken with permission from ref. 30).

6. HYBRIDIZATION METHODS FOR THE DETECTION OF CROSSLINKS IN SPECIFIC GENES

Most of the methods described above can separate crosslinked DNA molecules and, in some cases, quantitate and characterise the crosslinks formed in relatively small fragments of highly purified DNA. In addition, the hydroxyapatite chromatographic method can quantitate the levels of interstrand crosslinking present in the bulk of the genome in living cells. Similar results can be achieved very efficiently by alkaline elution. These latter methods, however, would be insensitive to local and limited heterogeneity of both crosslinking damage and its repair at the gene or sub-gene level in cells. A method has been developed to detect such crosslinking in specific gene sequences in cells. This was first described for psoralen crosslinking by Vos and Hanawalt [32,33]. The basic principle for the detection of the crosslinks remains the same as that described in Section 1, and the overall method is outlined in Fig. 8.

Genomic DNA is isolated and purified from drug-treated cells and restricted with an appropriate restriction enzyme. Following denaturation (by either alkali or heat) size fractionation of the DNA is achieved on a neutral agarose gel (0.4–2% depending on the size of the restriction fragments under analysis). After transfer to nitrocellulose or nylon membranes the specific gene fragments are identified by hybridization with the

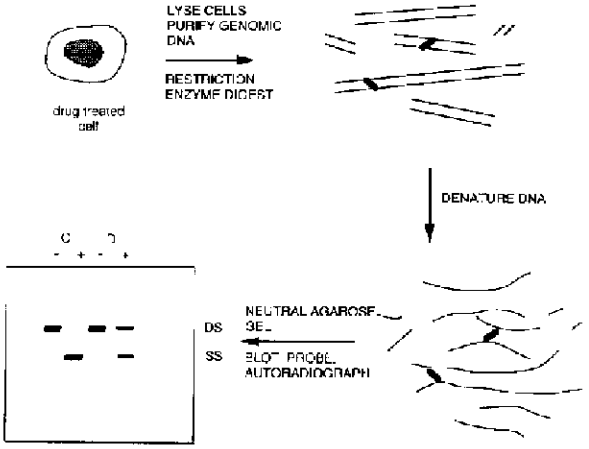

Fig. 8. Measurement of crosslinking in specific gene sequences in cells using a hybridization method. Genomic DNA from drug-treated cells is purified and digested with an appropriate restriction endonuclease. Following complete denaturation the DNA is fractionated on a neutral agarose gel, transferred onto nitrocellulose or nylon membranes, and the specific gene fragment identified by hybridization with the appropriate radiolabelled probe. In control samples (C) the fragment will appear as double-stranded (DS) if the denaturation step is omitted (−) or single-stranded (SS) following denaturation (+). The gene fragments containing interstrand crosslinking in the drug-treated sample (D) will run as double-stranded following the denaturation step.

appropriate radiolabelled probe. An example showing crosslinking in a 9-kilo base pair (kb) fragment of the human *n-ras* gene by the nitrogen mustard mechlorethamine is shown in Fig. 9. Densitometry of the resulting autoradiogram is then used to quantitate the proportion of DNA fragments containing crosslinks, and the mean number of crosslinks per fragment is calculated using a Poisson analysis. A detailed explanation of the methods of calculation and theoretical considerations are given in ref. 33.

The method is quite sensitive provided the blotting and hybridization are performed optimally with little non-specific background. For psoralen a sensitivity in the order of 0.1 crosslink per 30 kb genomic DNA (or one crosslink per $2 \cdot 10^8$ M_r) can be obtained [32] which is comparable to that of many other procedures used to quantitate crosslinks in bulk DNA. In the case of mechlorethamine a low level of crosslinking could be detected in the *c-myc* oncogene in human Colo320HSR tumour cells at a dose of drug which produces approximately 1 log of cell killing [34]. In order to obtain a reasonable level of crosslinking, however, super-toxic levels of drug are required.

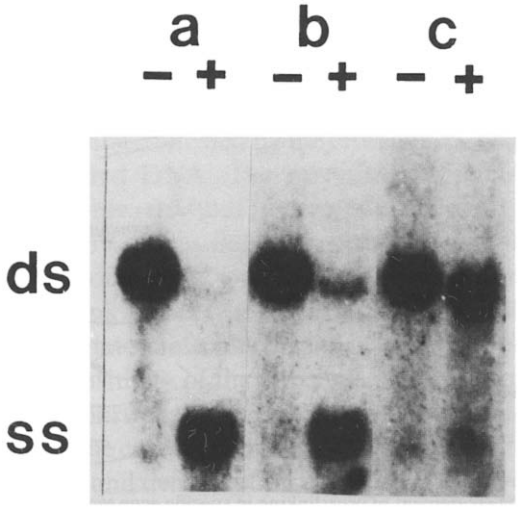

Fig. 9. Detection mechlorethamine-induced interstrand crosslinking in a 9-kb fragment of the human *n-ras* gene. Genomic DNA from human leukaemic K562 cells, cleaved with EcoRI, was size-fractionated on a neutral agarose gel either native (−) or following complete denaturation (+) and then transferred and hybridized with a labeled *n-ras* probe. The control non-drug treated samples (a) run as double-stranded (ds) and single-stranded (ss), respectively. (b) and (c) are drug treated with 10 μM and 100 μM mechlorethamine, and show the presence of dose-dependent crosslinking in the *n-ras* gene fragment as indicated by the double-stranded DNA in the denatured samples.

Repair of interstrand crosslinking at the gene level can also be followed by this method but several additional steps are required to obtain an accurate result. If the level of drug used does not inhibit DNA replication it is necessary to separate unreplicated (parental) DNA from DNA that has replicated during the repair period, otherwise this would lead to an inaccurate estimate of the repair efficiency. This is achieved using cell cultures in which the DNA has been pre-labelled with [^{3}H]thymidine. During the repair period non-labelled medium is used which contains bromodeoxyuridine (usually 10 μM) and fluorodeoxyuridine (1 μM) to incorporate density label into any newly synthesised DNA. Separation of the parental DNA can then be achieved using cesium chloride density gradient centrifugation prior to the denaturation, gel fractionation and hybridization steps.

7. PERSPECTIVES OF THE TECHNIQUES IN BIOMEDICINE

The chromatographic and, in particular, the electrophoretic methods outlined above, many of which have been developed recently, are powerful techniques for the separation of DNA molecules containing interstrand crosslinks. The neutral agarose gel-based technique is an efficient and sensitive *in vitro* method for determining the relative crosslinking efficiencies of different drugs. It has been used to re-evaluate the efficiency of crosslink formation of several classes of existing clinically used agents such as the nitrogen mustards [12], and to produce detailed kinetic data of the total and second-arm crosslink reaction not possible by other methods. For several chemotherapeutic nitrogen mustards the relative efficiency of crosslinking in isolated DNA determined by this assay correlated with the extent of crosslinking in cells determined by alkaline elution, and with the relative cellular cytotoxicities

of the drugs [35]. For agents of this type it is, therefore, a useful and relatively simple predictive assay of drug potency. In addition, it is becoming an extremely useful method for directly evaluating novel crosslinking agents with a potential clinical use such as the recently synthesised series of rationally designed DNA minor groove and sequence-selective linked anthramycin-based agents [36].

The polyacrylamide gel-based methods provide powerful techniques to examine the sequence selectivity of crosslink formation in duplex oligonucleotides. This provides often unexpected molecular information at the nucleotide level for existing drugs which can give clues to their mechanism of action. In addition, the analysis of novel agents can give important clues for rational design of sequence-specific drugs. For example, it has recently been demonstrated that the bioreductive alkylating agent 2,5-diaziridinyl-1,4-benzoquinone alters its preferred site of crosslinking following reduction from its quinone to its hydroquinone form [21], a feature that is now being incorporated in the design of more complex sequence-selective molecules.

With a combination of Sephadex chromatography and HPLC it is possible to obtain relatively large quantities of highly purified duplex oligonucleotides containing a single crosslink at a defined site. This will enable a sophisticated nuclear magnetic resonance (NMR) and X-ray crystallographic characterization of the crosslinked molecules. A detailed NMR analysis of a duplex containing a single mitomycin C crosslink purified in such a way has recently been reported [37]. Finally, the cellular analysis of damage by crosslinking drugs, and its repair, at the gene and subgene level utilising the hybridization methodology will allow for a much more detailed analysis of the molecular pharmacology of those crosslinking drugs which play an important role in cancer chemotherapy. It is already clear that mechlorethamine-induced DNA interstrand crosslinks are produced and processed in a heterogeneous fashion within the genome [34]. Such detailed analyses may give clues to the differing sensitivities of cells to such agents, the varying effectiveness of different crosslinking drugs, and may ultimately result in the rational design of more selective agents or protocols for clinical use.

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