Nucleic Acids Research, 1994, Vol. 22, No. 12 2311–2317

DNA damage by anti-cancer agents resolved at the nucleotide level of a single copy gene: evidence for a novel binding site for cisplatin in cells

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Received March 15, 1994; Revised and Accepted May 10, 1994

ABSTRACT

A new PCR based technique has been developed to investigate the sequence selectivity of adduct formation by DNA damaging agents in a single copy gene in isolated genomic DNA or in drug treated cells. Single-strand ligation PCR (sslig-PCR) demonstrated that cisplatin and nitrogen mustards reacted with guanine in an N-*ras* fragment with varying sequence specificity similar to that observed previously in plasmid DNA. In cisplatin-treated cells sslig-PCR demonstrated all the adducts found in isolated DNA and with the same sequence selectivity showing a preference for GG and AG sites. However, in cells an additional site of DNA binding of cisplatin was observed at the two occurrences of the sequence 5′-TACT-3′ on the transcribed and non-transcribed strands. This sequence is not a recognised target for cisplatin and represents a novel adduct formed in cells.

INTRODUCTION

The mechanism of action of many anti-cancer drugs and chemical carcinogens involves direct, covalent adduct formation on nucleotides in DNA. Nitrogen mustards, for example, alkylate mainly the N7 position of guanine residues, and cisplatin forms co-ordination complexes at the same position. Recently, the sequence preferences of lesion formation have been studied in isolated DNA (1) by exploiting the finding that such lesions can block the progression of DNA taq polymerases. This and other assays using plasmid DNA have revealed that even relatively simple DNA damaging agents such as cisplatin and the nitrogen mustards show a degree of sequence preference in adduct formation (2–5). Cisplatin forms mainly intrastrand cross-links between two adjacent guanines in runs of two or more consecutive guanines and between AG pairs (6,7). Most members of the nitrogen mustard class show a preference for runs of contiguous guanines, however some members show altered patterns of reactivity, e.g. quinacrine mustard reacts preferentially at the underlined guanine in 5′-GTPu and 5′-GGPu sequences (Pu = purine) (1,8).

Agents with a greater degree of sequence preference are being developed with the aim of producing more specific anti-cancer drugs and may open the possibility in the future for some degree of gene targeting. To aid the rational design of these drugs it is important to have accurate methods to determine their sequence specificity in a physiological environment. Studying their interactions in artificial systems with oligonucleotides or with plasmid DNA provides useful information but cannot predict their intracellular behaviour where DNA exists in a highly ordered structure, complexed with many proteins, and where other cellular components may affect reactivity. The lack of techniques to measure precise DNA binding within intact cells has also meant that the sequence selectivity of repair of individual lesions is a largely unexplored area. Southern blotting and PCR based methods currently available for the measurement of DNA repair in the intact cell can do so at the level of the gene (10–20 kb) (9,10) and regions within the gene (500–2000 bp) (11–13) respectively, but cannot give information at the level of the individual base.

To carry out such studies it is necessary to have a technique that is sensitive enough to measure DNA damage in a single copy gene within cells. DNA damage at the nucleotide level within cells treated with anti-cancer drugs has been investigated but these limited studies looked at the highly reiterated α-DNA and, while providing some useful information, have limited physiological significance (14,15). This methodology is not relevant to the study of single copy genes. A technique known as ligation mediated PCR (LM-PCR) has been recently developed whereby a double stranded oligonucleotide linker is ligated to blunt-ended DNA molecules formed by Sequenase extension of a primer, using as template DNA in which sites of alkylation or UV damage have been converted chemically or enzymatically into a strand break (16,17). The necessity for production of a stand break excludes its use for the study of many important anti-cancer drugs such as cisplatin, novel sequence specific agents and many carcinogens.

We have therefore developed and report here a method, single strand ligation PCR, which exploits both the observation that covalent DNA lesions can block taq polymerase (1), and the property of T4 RNA ligase to ligate (18) single stranded

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deoxyribo-oligonucleotides to single stranded DNA. We have shown that lesions can be demonstrated at the level of individual bases in intact cells treated with cisplatin. While most lesions were the same, an unexpected additional binding site was revealed in drug treated cells that was not seen in isolated drug treated DNA.

MATERIALS AND METHODS

Drugs

Cisplatin was obtained from Lederle as a 3.3 mM injectable solution and diluted to working concentrations in H_2O. Uracil mustard (Upjohn Corporation) and quinacrine mustard (Fluka) were freshly prepared as 10 mM solutions in DMSO and further diluted in H_2O to working concentrations.

Cells and cell culture

U937 Cells were grown in suspension at 37°C with 5% CO_2 in Iscoves modified DMEM tissue culture medium supplemented with 10% heat inactivated foetal calf serum.

Oligonucleotides

From the transcribed strand
3.1: 5′ CAG CAA GAA CCT GTT GGA AAC CAG 3′
3.2: 5′ CCA GTA ATC AGG GTT AAT TGC GAG C 3′
3.3: 5′ GCG AGC CAC ATC TAC AGT AC 3′

From the non-transcribed strand
5.1: 5′ GGT CCT TCC ATT TGG TGC CTA CG 3′
5.2: 5′ ACG TGG GGA GAT CTT GGA GA 3′
5.3: 5′ TGG AGA CAG AAG GGA GAA TG 3′

Ligation olignucleotide
5′ ATC GTA GAT CAT GCA TAG TCA TA 3′

'Upstream' primer
5′ TAT GAC TAT GCA TGA TCT ACG AT 3′

Primers 3.1 and 5.1 which were 5′ biotinylated and the ligation oligonucleotide which bears a 3′ primary amine and a 5′ phosphate group were supplied by Genosys UK. Other primers were supplied by Genosys UK or R & D Systems Europe Ltd.

Drug treatment of isolated DNA

Aliquots (3 μg) of genomic DNA (isolated from U937 cells) were incubated with or without drug for one hour at 37°C in TeOA (25 mM triethanolamine, 1 mM EDTA, pH 7.2) in a total volume of 50 μl. DNA was precipitated with ethanol, washed twice with 70% ethanol, dried and resuspended in 20 μl H_2O for sslig-PCR.

Drug treatment of cells

1×10^6 U937 cells, washed free of foetal calf serum, were treated with drug, for the times indicated in the figure legends, in a total volume of 1 ml Iscoves modified DMEM in 24 well plates (Falcon) at 37°C and 5% CO_2.

DNA preparation from cells

After drug treatment cells were transferred to 1.5 ml microfuge tubes, washed 3 times with 1 ml PBS and resuspended in 340 μl lysis buffer (400 mM Tris−HCl pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% w/v SDS) to which was added 100 μl 5 M sodium perchlorate. After vortexing, the suspension was incubated with shaking at 37°C for 20 minutes, then at 65°C for 20 minutes with occasional agitation. 580 μl of chloroform pre-cooled at −20°C was added and the mixture was rotated for 20 minutes at room temperature. After centrifugation for 10 minutes at top speed in a microfuge (MSE) half of the DNA containing upper layer (220 μl, equivalent to 5×10^5 cells) was removed and the DNA precipitated with 440 μl absolute ethanol, washed twice with 70% ethanol, dried, resuspended and restricted overnight with 6 units of *Pvu*II or *Hin*dIII (12 units/μl, NBL, UK) in a volume of 20 μl. The digested DNA was precipitated and resuspended in 20 μl H_2O. This DNA was then subjected to sslig-PCR.

Sslig-PCR

1st round 'linear' PCR was carried out, in a volume of 100 μl, using 3 μg of drug treated DNA, or DNA isolated from the equivalent of 5×10^5 cells. The PCR was carried out using 0.6 pmoles of 5′-biotinylated primer 3.1 or 5.1 and the reaction mixture was composed of 20 mM $(NH_4)_2SO_4$, 75 mM Tris−HCl pH 9.0, 0.01% (w/v) Tween 20, 6.25 mM $MgCl_2$, 0.01% gelatin, 250 μM each dNTP and 1 unit taq polymerase for the non-transcribed strand and the same for the transcribed strand except that the $MgCl_2$ concentration was 4 mM. The DNA was initially denatured at 94°C for 5 minutes and then subjected to 30 cycles of 94°C, 1 min, 60°C, 1 min, 74°C, 1 min + 1 second extension per cycle on a PT-100 thermal cycler with hot bonnet (MJ Research, USA). The mixture was finally incubated at 94°C for five minutes and then cooled to 4°C. It is important to optimise the $MgCl_2$ concentration in this step for each new set of primers as it can influence the efficiency of the reaction. To capture and purify the products of biotinylated primer extension the PCR mixture was precipitated and resuspended in 30 μl binding buffer (5 mM Tris−HCl pH 7.5, 1 mM EDTA, 1 M NaCl) to which was added 5 μl washed streptavidin Dynabeads (Dynal UK). The suspension was incubated for 30 minutes at room temperature with occasional agitation. The beads were sedimented in a magnetic rack and washed 3 times with 200 μl 10 mM TE pH 7.6. The beads were resuspended in 10 μl ligation mixture containing 10 units T4 RNA ligase (20 units/μl, New England Biolabs) in 50 mM Tris−HCl pH 8.0, 10 mM $MgCl_2$, 10 μg/ml bovine serum albumin, 1 mM hexammine (III) cobalt chloride, 20 μM ATP, 25% (w/v) PEG 8000, 20 pmol of gel purified ligation oligonucleotide and ligated overnight at 22°C. The ligation oligonucleotide was supplied with a 5′ phosphate, essential for ligation, and a 3′ terminal amine which blocks its self-ligation. After ligation the beads were washed 3 times with 200 μl TE pH 7.6 and resuspended in 40 μl H_2O for the second round PCR. The second round PCR mixture, the volume of which was 100 μl, contained 10 pmol each of primer 3.2 or 5.2 and the gel purified 'upstream' primer which was complementary to the ligated oligonucleotide. The buffer composition was as for first round PCR except that 2.5 units of taq polymerase were used and the magnesium concentration was 5.0 mM for the non-transcribed strand and 2.5 mM for the transcribed strand. The cycling conditions were: an initial denaturation at 94°C for five minutes then 23 cycles for the non-transcribed strand and 27 cycles for the transcribed strand of 94°C, 1 min, 58°C, 1 min, 74°C, 1 min + 1 sec extension per cycle. The third round PCR was carried out by adding 10 μl of ^{32}P 5′ end-labelled primer 3.3 or 5.3 in PCR reaction buffer containing 5 pmol labelled primer and 1 unit taq polymerase. The mixture was subjected to 3 further cycles of

94°C, 1 min, 60°C, 1 min, and 74°C, 1 min. The reaction mixture was precipitated with ethanol, resuspended in 5 μl formamide loading buffer, denatured at 95°C for three minutes, cooled on ice and electrophoresed at 2500–3000 volts for three hours in a 80 cm×20 cm×0.4 mm, 6% acrylamide sequencing gel (Sequagel, National Diagnostics). The gel was dried and autoradiographed (Hyperfilm, Amersham).

RESULTS

Single strand ligation PCR (sslig-PCR)

The method of sslig-PCR is outlined in Figure 1. It involves a first round PCR using a single 5′-biotinylated primer which defines the area of the gene to be investigated. 30 cycles of linear amplification by PCR generates a family of single-stranded molecules of varying length for which the 5′- end is defined by the primer and for which the 3′- ends are defined by the positions of the DNA–drug adducts without the need for a strand break. In order to exponentially amplify these molecules, which are captured and isolated by binding to streptavidin coated magnetic beads, a single stranded, 5′-phosphorylated, oligonucleotide is ligated to their 3′-OH ends using T4 RNA ligase (18). With both ends of the DNA molecules defined they can then be exponentially amplified and detected. The use of 'linear' PCR in the first primer extension should increase its sensitivity compared to LM-PCR which is limited to one extension reaction of the first primer to produce a double stranded blunt ended molecule. We have found single-stranded ligation PCR (sslig-PCR) to be a sensitive and reproducible technique and we have applied it to study the interaction of various anti-cancer drugs with a region of a single copy gene, namely intron 1 of N-*ras* (19), in human-derived cell lines.

Drug binding to isolated genomic DNA

Cisplatin, when incubated with 'naked' genomic DNA, bound preferentially to runs of two or more consecutive guanines and to AG pairs (Fig. 2a) and adducts were detected at all occurrences of these sequences. Cisplatin was also observed to bind to the first guanine in the sequence 5′-GCG (data not shown). The reproducibility of the method is demonstrated in lanes C and D which represent two independent drug treatments.

Sequence preference of binding of uracil mustard and quinacrine mustard is compared to cisplatin in Figure 2b. The genomic DNA used as template for these reactions had been previously digested with *Pvu*II which cuts 358 bp upstream of the end-labelled primer and thus creates a defined stop site for taq polymerase on lesion-free templates. In drug treated DNA the intensity of the band at this 'full-length' site is substantially reduced due to downstream blockage of taq polymerase by lesions. Uracil mustard preferentially alkylates the N7 position of guanines and as demonstrated in Figure 2b its pattern of adduct formation shows some differences to cisplatin. It binds with less intensity to runs of guanines but binds strongly to some single guanines when followed by cytosine. Quinacrine mustard also alkylates guanines and was found to have a particular preference for 5′GGpu and 5′GTpu sites. The pattern of adduct formation by these agents identified by sslig-PCR in genomic DNA was identical to that previously observed in plasmid DNA (1,3). The exponential amplification involved in sslig-PCR therefore does not affect its accuracy in identifying individual lesions. These experiments show that sslig-PCR can be used to resolve DNA damage at the individual nucleotide level of a single copy gene in isolated genomic DNA. The method was therefore applied to DNA isolated from drug treated cells to determine sequence specificities in the cellular environment, the conditions of which could influence adduct formation by anti-cancer drugs and by other DNA damaging agents including many carcinogens.

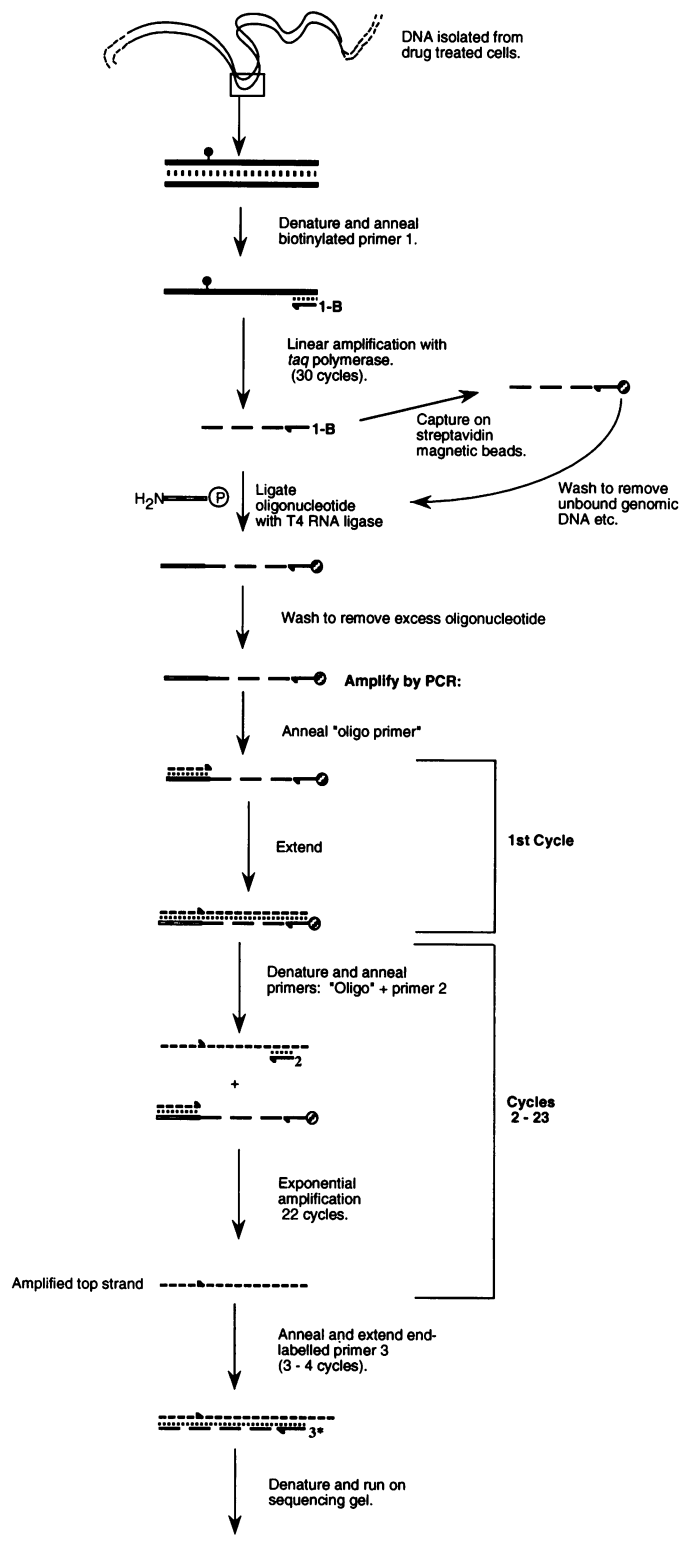

Figure 1. sslig-PCR method.

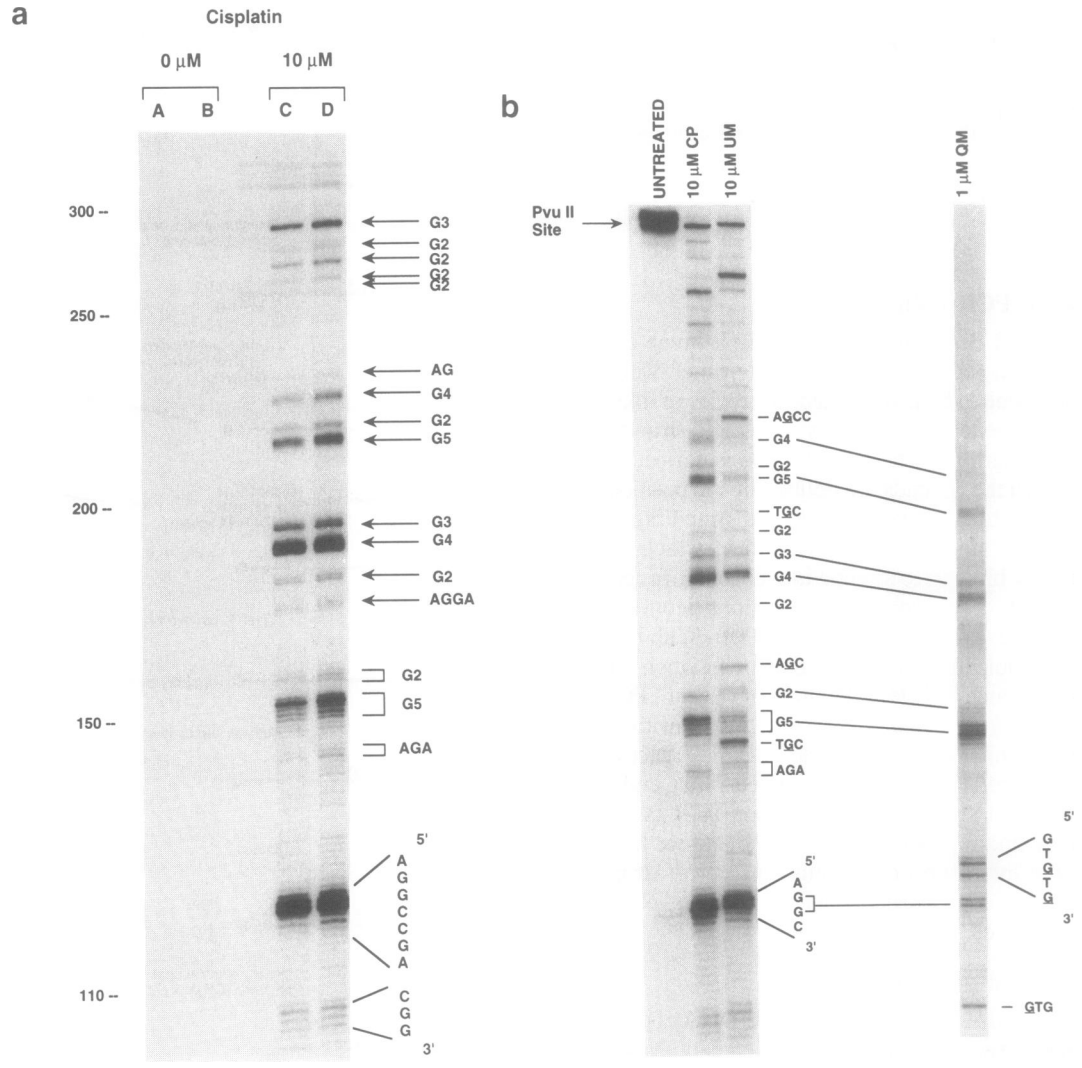

Figure 2. (a) Sslig-PCR on the non-transcribed strand using isolated genomic DNA treated with 10 μM cisplatin. The lanes A + B and C + D show the results from duplicate treatments of DNA either with or without cisplatin. The figures on the left of the diagram refer to the distance in bases from the first base of the end-labelled primer (primer 3.3). The letters on the right indicate the bases which correspond to the bands on the gel. G2, G4, G5, etc. represent runs of consecutive guanines. (b) Sequence selectivity of adduct formation of uracil mustard (UM) and quinacrine mustard (QM) compared to cisplatin (CP) in *Pvu*II digested genomic DNA. Underlined bases indicate the position of the lesion.

DNA binding specificities of cisplatin in cells

Figure 3 shows the results obtained, from both the non-transcribed and transcribed strands, with cells treated with cisplatin. Higher doses of drug are needed since less drug reaches the DNA compared to drug treated isolated DNA. With the non-transcribed strand as template some background bands appear in the untreated control which seem to represent intrinsic obstructions to taq polymerase since they consistently appear at the same positions. They may be due to some secondary structure of the DNA, especially in GC base pair regions, blocking to some extent the progress of the taq polymerase in the first round of PCR. However all attempts to overcome the effects of secondary structure, such as the inclusion of co-solvents DMSO, formamide or spermidine, or pre-denaturation with sodium hydroxide failed to remove these bands. Interestingly the background when the transcribed strand is used as template for sslig-PCR is consistently very low and the overall level of damage at a given dose of drug, as indicated by the decrease in 'full-length' product, is also lower than on the non-transcribed strand(Fig. 3b).

With the non-transcribed strand specific bands, both quantitatively and qualitatively different from the control, are seen when DNA from drug treated cells is used. This can be seen clearly for cisplatin (Fig 3a), with the intensity of the bands showing a dependence on drug concentration. As expected, the intensity of the 'full-length' band is highest in untreated cells and decreases as the concentration of cisplatin increases. It can also be seen that the intensity of individual lesions increases from 50 μM to 100 μM cisplatin (e.g. the 5'-AGG-3' sites at position 121−123), but when the concentration is increased to 200 μM the intensity of the bands closest to the primer increase whereas those further away from the primer either show no change (5'-AGG-3' at position 121−123) or actually decrease in intensity (e.g. 5'-TACT-3'). As the concentration of drug increases the percentage of template molecules bearing more than one lesion will increase and the taq polymerase will be blocked at the first

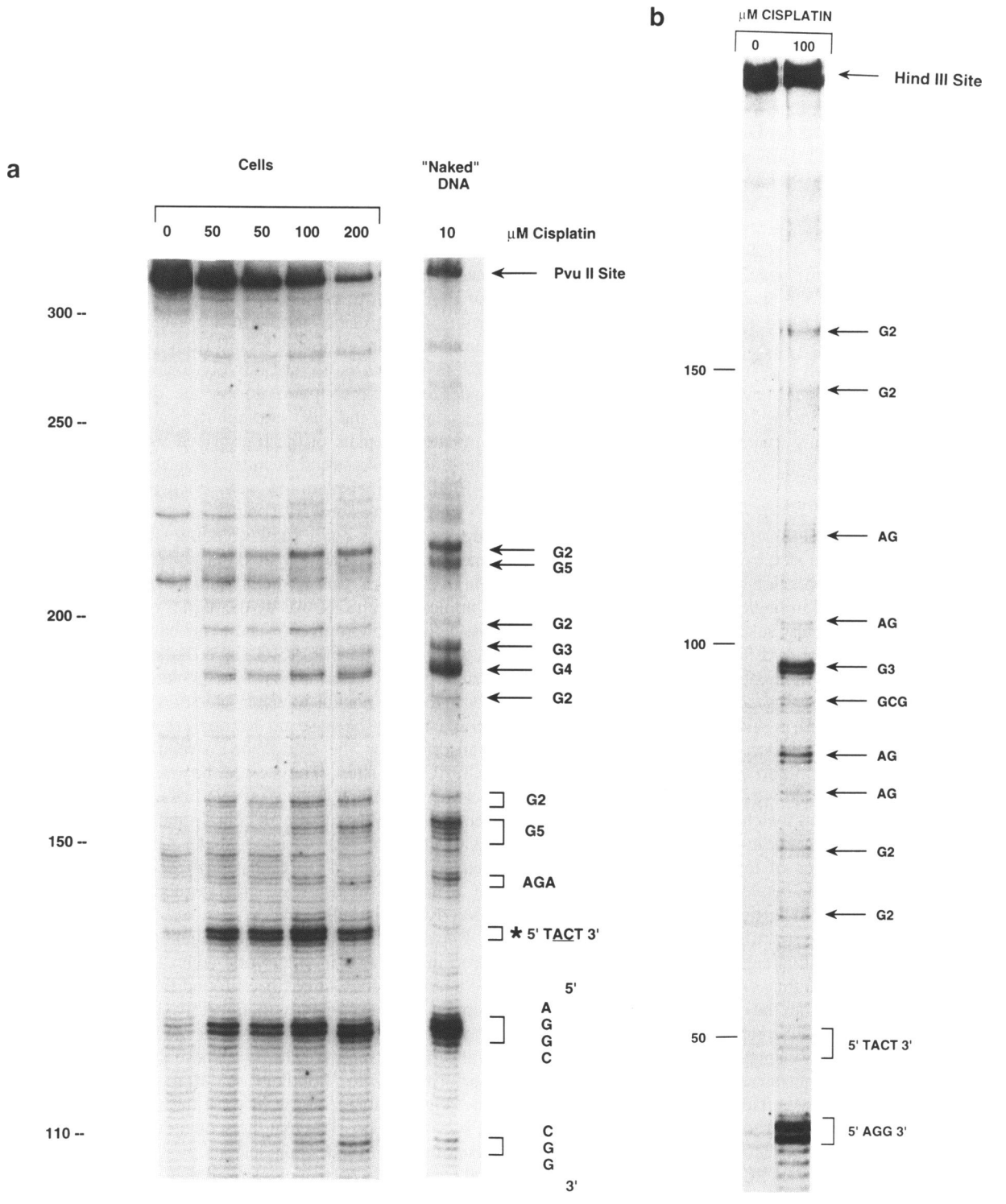

Figure 3. (**a**) Concentration dependent adduct formation on the non-transcribed strand by cisplatin in cells and comparison with treatment of 'naked' DNA. The bands on the autoradiograph represent cisplatin adducts formed in cells that were treated with the indicated concentrations of cisplatin for 18 hours. DNA extracted from cells was cut with PvuII before sslig-PCR to create a defined stop site on lesion free templates. The asterisk next to the sequence 5'-TACT indicates lesion sites which were cell specific as adducts were not detected at these positions in 'naked' DNA. (**b**) Cisplatin adduct formation in cells on the transcribed strand. Before sslig-PCR the DNA extracted from cells was digested with *Hin*dIII which cuts at a site 272 bp downstream of the primer 5.3 binding site.

lesion, i.e. the one closest to the primer. Figure 3a also demonstrates the reproducibility of the method in cells, the intensities of individual bands in duplicate drug treatments being very similar as can be seen with the two independent 50 μM treatments.

The sequence specificity of cisplatin binding in cells is generally

very similar to that in isolated genomic DNA (see Fig. 3a) and adducts are formed at all runs of two or more guanines. In the run of 5 guanines at position 151−155, in both 'naked' DNA and in treated cells the fourth guanine at position 154 produces a more intense band than the other four. The site of strongest binding in both cells and naked DNA is at the sequence 5′-AGG-3′ at position 121−123. However bands of almost equal intensity are seen in cells at the site 5′-TACT-3′ which are not present at all in treated naked DNA. This finding, which is highly reproducible, therefore represents a novel, cell-specific, lesion. The sequence in this region, 5′-TACT-3′, occurs only once on the non-transcribed strand in the gene segment under study. It can be seen in Fig. 3b that the most intense bands on the transcribed strand are also at the sequence 5′-AGG-3′. Binding is also detected at the unique 5′-TACT-3′ on the transcribed strand which although of much less intensity compared to the non-transcribed strand, is of equal intensity compared to other GG pairs on this strand. This finding is consistent and no bands appear at these positions on drug treated naked DNA (data not shown).

DISCUSSION

This novel method allows the determination of the sequence specificity of covalent adduct formation by DNA damaging agents at the individual nucleotide level in single copy genes. The method is sensitive, so that physiologically relevant doses of drug could be used both with isolated DNA and for the treatment of intact cells. This method should be applicable to any molecule that covalently binds to DNA creating a lesion that blocks taq polymerase. It can be used with a wide range of agents since it is not limited to those whose lesion can be converted into a strand break. It should be possible to use sslig-PCR with many types of chemical carcinogens and we have used it to detect UV induced lesions (data not shown) which are also known to block taq polymerase (11). Therefore studies are now possible at the cellular level to determine sites of lesions and to reveal, for example, if there is a correlation between adduct formation and the positions of base mutations in particular genes. Furthermore in the development of sequence specific drugs it is important to be able to establish whether within cells these agents do indeed bind to the sequences for which they were designed. Studies on the behaviour of such drugs within the cellular environment may provide information to aid in their rational design.

The sequence specificities for cisplatin detected by sslig-PCR are in accordance with the intrastrand crosslink formation behaviour of cisplatin previously elucidated using plasmid DNA (1,4,5,20). It is likely that all cisplatin adducts formed are detected by sslig-PCR. A quantitative PCR assay has demonstrated that taq polymerase is completely blocked by one adduct per DNA strand (13), and Comess *et al.* (20) have shown that, in artificial systems, adduct bypass by taq polymerase is at most 3% for GG crosslinks and 19% for AG crosslinks. It can be seen that adducts on pairs of guanines are represented by two bands on the autoradiograph whereas only one might be expected. One interpretation is that this is due to the presence of monoadducts at the guanine sites. This is unlikely however, for several reasons. It has been demonstrated in both isolated DNA and in cells that intrastrand crosslinks (both GG and AG) account for more than 90% of lesions on DNA (6, 21). Such intrastrand crosslinks form rapidly (22) and are likely to be the lesions detected by sslig-PCR at the GG sites since similar patterns of binding were observed with isolated DNA following 1 hour or up to 18 hours of incubation (data not shown). Two bands have also been demonstrated in plasmid DNA containing a single crosslink (20) and are probably due to greater distortion of the DNA on the 5′ side of the cross-link than on the 3′ side (23−25). The 3′ adduct of the cross-link would then only partially block taq polymerase occasionally allowing bypass and subsequent blockage at the 5′ adduct.

In cells a consistent background of bands appears in the untreated controls. These may be due to spontaneous DNA damage at labile sites (26). The background was much less when the transcribed strand was used as template. A possible reason for this is that transcription coupled repair is removing lesions from the transcribed strand at a greater rate than other repair mechanisms are removing lesions from the non-transcribed strand (27,28). N-*ras* is a constitutively expressed gene and will be undergoing transcription during the drug treatment. Such a mechanism could also explain the lower background in the untreated template of the transcribed strand if that seen on the non-transcribed strand is indeed due to endogenous damage.

Binding of cisplatin to both occurrences of the sequence 5′-TACT-3′ was observed on the transcribed and non-transcribed strands. This is not a known binding site for cisplatin and was unexpected. It was anticipated that some lesions might be absent in DNA from treated cells due to obstruction by proteins complexed with DNA or by the higher order of structure within the cellular environment. The extra bands are not the result of a point mutation of C to G since the same cells were also the source of the 'naked' DNA. The blockages at these 5′-TACT-3′ sequences are not due to either interstrand cross-links or to double strand breaks because no lesions are seen in either the non-transcribed or the transcribed strand at bases opposite the 5′-TACT-3′ sites. They could be the result of cross-linking between DNA and nuclear protein in these regions. Alternatively the local DNA structure in cells is such that it allows cisplatin to bind to cytosine as well as adenine. Although the interpretations remain hypothetical the results clearly demonstrate the importance of a method to determine the sequence binding specificities directly within cells.

Repair of drug damaged individual bases in genes has not yet been investigated in eukaryotic cells. DNA repair is known to be heterogeneous, it has been found that active genes are often repaired more efficiently than inactive genes, (29−31) and that the transcribed strand is repaired more rapidly than the non-transcribed strand (27,32,33). These studies have mainly been performed with Southern blotting based methods which investigate damage at the level of the whole gene (10−20 kb). It is however possible that the sequence environment affects the rate of repair of individual lesions. For example, in the bacterial *lac* I gene it has been demonstrated that, after UV damage, lesions at certain sites are repaired slowly and that these repair 'slow spots' correlate with the positions of mutation 'hot spots' (34). Thus the precise position of genetic mutations caused by carcinogens may be determined by both the distribution of the initial damage and by the efficiency of the subsequent repair of individual lesions. It is important from the point of view of carcinogenesis to determine if similar correlations exist in mammalian cells. In this study cells were treated with cisplatin for 18 hours. For repair experiments shorter treatments will be necessary so that the cells remain viable. Preliminary work has shown that after treatments of 2 hours and 5 hours in cells the total binding of cisplatin is less but the binding patterns obtained

are identical to those presented in this study, including at the novel 5′-TACT-3′ site, (data not shown). Furthermore cells treated with cisplatin for 5 hours at concentrations between 50–200 μM are capable of subsequent repair as determined by a quantitative PCR assay (unpublished observations). Therefore sets of primers from each strand it will be possible to use sslig-PCR to compare damage and repair at the nucleotide level in the transcribed and the non-transcribed strand.

Repair studies at the nucleotide level are also particularly important in the investigation of anti-cancer drugs. For example Zhen *et al.* (35), using Southern blotting, have found differences in the gene specific repair of cisplatin interstrand, but not intrastrand, crosslinks which seemed to correlate with the degree of resistance to the drug. Further studies on nucleotide-specific lesions will help to identify molecules capable of creating lesions which are resistant to repair.

ACKNOWLEDGEMENT

This work was funded by a project grant from the Cancer Research Campaign (SP 2000/0301).

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