Construction of a Human Shuttle Vector Containing a Single Nitrogen Mustard Interstrand, DNA-DNA Cross-Link at a Unique Plasmid Location

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ABSTRACT

DNA cross-linking reagents are frequently unusually cytotoxic, and many, including the nitrogen mustards, are potent chemotherapeutic agents, presumably because DNA cross-links effectively block DNA replication. Most of these reagents form both inter- and intrastrand DNA cross-links, but it is unknown which is more effective at blocking replication and why. To evaluate the role of interstrand cross-links, a human shuttle vector was constructed that contains a single, nitrogen mustard interstrand cross-link at a unique site. In previous work (J. O. Ojwang, D. A. Grueneberg, and E. L. Loechler, Cancer Res., 49: 6529-6537, 1989) a duplex oligonucleotide was synthesized that had an interstrand cross-link derived from a nitrogen mustard moiety bound at the N(7)-position of the guanines in the opposing strands of a 3'-GAC-5' sequence. Herein, a procedure is described to incorporate this oligonucleotide into an SV40-based human shuttle vector, which was designed for these experiments. The purified cross-linked vector was characterized and shown: (a) to have a chemical (i.e., a nitrogen mustard) modification at the anticipated genome location; (b) to have a modification that covalently joins the two duplex strands of the vector together; and (c) to contain a single interstrand cross-link per genome. The methodologies described to construct this vector are expected to be generally applicable and, thus, site-specific incorporation of an interstrand cross-link derived from any appropriate chemical should be possible. These procedures complement existing methodologies that permit the incorporation of monoadducts and intrastrand cross-links into vectors in a site-specific manner.

INTRODUCTION

It has become clearer in recent years that many toxic and/or carcinogenic chemicals have DNA as a target, and they frequently form covalent DNA adducts (1). These adducts are of a diverse nature, which is reflected in the myriad of ways by which they are processed by the cell; e.g., adducts may differ in their mutagenic and/or cytotoxic potential (2), and in their mode of DNA repair (3). The study of the biological consequences of individual DNA adducts has advanced recently because of the development of techniques to construct DNA vectors that contain adducts of defined chemical structure at known genome locations (4). A general strategy has been followed in virtually all of these studies (4–12); a single-stranded oligonucleotide that contains a specific adduct is synthesized and, subsequently, incorporated into an appropriate vector by using recombinant DNA techniques. One type of DNA lesion, the DNA-DNA interstrand cross-linked, cannot in most cases be incorporated into DNA vectors by the procedures outlined above, which utilize single-stranded oligonucleotides containing adducts of defined structure. One exception to this statement involved an elegant set of experiments where an Escherichia coli vector, which was constructed with a site specifically incorporated, interstrand cross-link derived from a psoralen, was used to study the biological consequences of the cross-link (11). However, the method used in this study (11) is not generally applicable because a single-stranded oligonucleotide that contained a psoralen monoadduct was synthesized (12) and incorporated into a vector by procedures alluded to in the previous paragraph (4–12), and then the interstrand cross-link was generated from the monoadduct by UV irradiation of the vector (11).

The study of interstrand cross-links is particularly relevant to the mechanisms of action of many anticancer alkylating agents, which are frequently DNA cross-linking reagents, an activity that is essential for their effectiveness (13–15). DNA-protein cross-links appear not to be correlated with cytotoxicity (16, 17). However, the role of DNA-DNA interstrand versus intrastrand cross-links in causing cytotoxicity, presumably via blocking DNA replication, is less clear. Interstrand cross-links have been implicated in the case of nitrosoureas (16–21), while intrastrand cross-links have been implicated in the case of other chemotherapeutic agents, such as cis-diamminedichloroplatinum(II) (22). Less definitive information in this regard is available for the important class of chemotherapeutic agents, the nitrogen mustards, which are known to form cross-links (23, 24) that are both interstrand (25, 26) and, in all likelihood, intrastrand by analogy to the reactions of sulfur mustards (27). Interstrand cross-links are also derived from a variety of other chemicals, including chloroacetalddehyde and malondialdehyde (28, 29).

To investigate the role of inter versus intrastrand cross-links in blocking DNA replication and, presumably, in causing cytotoxicity, we have initiated a project to build both classes of lesions into DNA vectors in vitro in order to study the ability of each to inhibit the synthesis of progeny DNA molecules and to induce mutations (30). Herein, we describe the incorporation of a duplex oligonucleotide, which contains a single HN2 interstrand cross-link (30), into an SV40-based shuttle vector, and the characterization of the product, inter-HN2-pTZSV28.

MATERIALS AND METHODS

Nitrogen mustard is an extremely dangerous compound and was handled with great care as described previously (30). T4 DNA polymerase, DNA polymerase I large fragment (Klenow), T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were obtained from New England Biolabs. pTZ19R and calf intestinal phosphatase were obtained from Pharmacia. Human 293 cells (31) were a gift from Dr. M. Calos, Stanford University, Stanford, CA.

Shuttle Vector Construction

The human shuttle vector, pTZSV28 (Fig. 1), was constructed from the early region of SV40 (which includes both SV40 enhancers, the origin of replication, and genes for both the small and large T-antigens) and the pBR322 derivative, pTZ19R.4 pTZ19R has both an H1 and a

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3 The abbreviations used are: HN2, nitrogen mustard; M1 and X1, dodecameric oligonucleotides (Fig. 1); 1-M1-HN2-X1, a modified oligonucleotide with M1 and X1 covalently joined by a nitrogen mustard interstrand cross-link as described in the text; inter-HN2-pTZSV28, a vector containing a nitrogen mustard interstrand cross-link; C-pTZSV28, a vector identical to inter-HN2-pTZSV28 but lacking the cross-link; TE, buffer described in Ref. 33.

4 pTZ19R is a vector constructed by Pharmacia that is similar to pUC19 (32).
ColEI origin of replication, a bla gene, and a lacZ' gene with a polynucleotide. By standard procedures (33) the early region of SV40 was isolated on a 3004-base pair (KpnI to BamHI) fragment, blunt ended, and incorporated into the PBR322 derivative, pTZ19R, at positions 1273 (Hgal), 2288 (SpiI), or 2841 (SpiI) to give pTZSV12, pTZSV22, and pTZSV28, respectively. pTZSV28 was chosen because, following transfection (33) into human 293 cells (31), incubation for 48 h and isolation of replicated plasmids by the Hirt procedure (35), it gave the highest yield of ampicillin-resistant colonies upon transformation of E. coli, MM294A, cells (−10⁵ transformants/μg used in the original transfection). To ensure that only plasmids replicated in human 293 cells were transformed, the Hirt supernatant was treated with DpnI prior to transformation (31, 36). Unfortunately, the fl origin in pTZSV28 is inactive and no replication-dependent recognition site was added between the HindIII and the SpiI site in the polynucleotide region of pTZSV28 (Fig. 1). The PstI site in the large T-antigen gene of pTZSV28 was eliminated by changing a wobble base using site-directed mutagenesis (38).

Construction of Inter-HN2-pTZSV28

1-M1-HN2-X1 (Ref. 30; see legend to Fig. 1) was incorporated into pTZSV28 to give a modified vector, designated inter-HN2-pTZSV28, by a 5-step procedure (Fig. 2). A control vector containing no cross-link (designated C-pTZSV28) was also constructed.

Step 1. pTZSV28 (10 μg) was digested with MluI (16 units for 2 h at 37°C) to give a genome length, linear vector, which was deproteinized with phenol/chloroform, ethanol precipitated, and resuspended in TE buffer (10 μl). (Phenol extraction is performed to remove MluI, which could reverse step 2.)

Step 2. Linearized pTZSV28 (10 μg in 10 μl) was ligated with the cross-linked oligonucleotides, 1-M1-HN2-X1 (200 ng, a 10-fold molar excess, in 2 μl), using T4 DNA ligase (100 units, for 2 h at 16°C in 50 mM Tris (pH 7.5), 10 mM MgCl₂, 20 mM dithiothreitol, and 1 mM ATP; final volume, 25 μl). In this step 1-M1-HN2-X1 had a 5′-phosphate ([32P]-radiolabeled) on its MluI overhanging end, but a 5′-hydroxyl group on its XbaI overhanging end, which ensured that the product had only one 1-M1-HN2-X1 moiety incorporated on each end.

Step 3. The product from step 2 was cleaved with XbaI (40 units, 2 h at 37°C), which removed a small fragment that contained one of the two oligonucleotides and exposed the internal XbaI site in the polynucleotide region of the vector. The larger fragment was isolated from an agarose gel by using the glass powder (GENECLEAN, BIO 101) absorption method (Ref. 39; recovery, ~70%) and ultimately resuspended in TE buffer (50 μl). (Phenol extraction is performed in this step to remove MluI, which could reverse the reaction performed in step 3.)

Step 4. The product of step 3 was phosphorylated on the XbaI overhanging end that was originally associated with the oligonucleotide, using T4 polynucleotide kinase (20 units) and [γ-32P]ATP (3.4 pmol; specific radioactivity, 3000 Ci/mmole) for 1 h at 37°C. Although there was no indication that this reaction did not go to completion, a chase with unlabeled ATP was conducted. ATP (50 nmol) and additional T4 polynucleotide kinase (10 units) were added and incubated an additional 30 min at 37°C (final conditions: 166 mM Tris, 17 mM MgCl₂, 8 mM dithiothreitol, pH 7.6; final volume 60 μl).

Step 5. The phosphorylated, linear product from step 4 was ligated to give closed circular DNA as follows. The DNA was diluted with sterile water to a concentration of 1 mM/ml (final volume, 7 ml) and redistributed into 7 Eppendorf tubes; this low DNA concentration ensured that intramolecular ligation predominated. T4 DNA ligase (100 units) was added to each tube for 2 h at 16°C (50 mM Tris (pH 7.5), 10 mM MgCl₂, 20 mM dithiothreitol, and 1 mM ATP). The samples were deproteinized with phenol/chloroform, ethanol precipitated, which was facilitated by the addition of 5 μg of polyacrylamide carrier, and resuspended in TE (total volume, 50 μl). The cross-linking vector is designated inter-HN2-pTZSV28, and the control with no cross-link is designated C-pTZSV28.

After the procedure outlined in Fig. 2, closed circular material was purified as follows. Inter-HN2-pTZSV28 was shown to be insensitive to cleavage by AccI, whose activity was superior to SafI, due to the presence of the cross-link in its AccI/SafI recognition site (see below), and it was treated with AccI to linearize any contaminating material that might not contain a cross-link. Cesium chloride density gradient centrifugation was used to purify closed circular DNA away from both linear and open circular DNA (33). A hole was punctured in the bottom of the centrifuge (Beckman) tube with a 20-gauge hypodermic needle, and ~0.15-ml fractions were collected into Eppendorf tubes; fractions containing closed circular DNA were identified by Cearex counting. Aliquots from fractions containing radioactive electrophoresed through an agarose gel, which was dried, and autoradiography was performed (Fig. 3). Fractions containing closed circular material were identified (Fig. 3) and pooled (~0.45 ml), extracted 3 times with n-butyl alcohol, ethanol precipitated (washed once in 70% ethanol), resuspended in 100 μl of TE (10% glycerol), and stored at 20°C. On different occasions, between 0.2 and 1 μg of closed circular DNA containing the cross-link (inter-HN2-pTZSV28) was isolated (yield, ~2 to 10%).

Characterization of Inter-HN2-pTZSV28

Following cesium chloride purification, closed circular inter-HN2-pTZSV28 and C-pTZSV28 (~25 ng, ~1000 cpm in each case) were shown (Fig. 4) to be sensitive and sensitive, respectively, to cleavage with AccI (10 units, 1 h at 37°C). Inter-HN2-pTZSV28 and C-pTZSV28 were mixed with pTZSV28 itself (200 ng, unlabeled) and treated with PstI (1 unit, 1 h at 37°C); pTZSV28 was linearized as visualized by ethidium bromide staining following agarose gel electrophoresis, while inter-HN2-pTZSV28 and C-pTZSV28 were not cleaved as visualized by autoradiography (Fig. 4). Unlabeled pTZSV28 was included as a carrier because of nonspecific nicking that occurred with PstI.

To assess if inter-HN2-pTZSV28 contained an interstrand cross-link, a denaturation/renaturation experiment was performed (rationale in "Results"). Purified, closed circular inter-HN2-pTZSV28 (~15 ng in 6 μl; ~600 cpm) was linearized with BamHI (20 units for 1 h at 37°C), was incubated with 1 μl of 0.5 M EDTA (pH 8.0) for 2 min, and was denatured by the addition of NaOH (3 μl of a 1 N solution) for 1 min; immediately thereafter, 2 μl of loading dye (0.25% bromophenol blue, 40% sucrose) were added and the sample was electrophoresed in a 0.8% agarose gel for 2 h at 100 V. Renaturation occurred in the well of the agarose gel. The gel was dried and autoradiography was performed.

Provided the strategy outlined in Fig. 2 was successful, inter-HN2-pTZSV28 should contain a single copy of 1-M1-HN2-X1, which was established by showing that the polynucleotide region of inter-HN2-pTZSV28 was of the correct size (Fig. 6). Inter-HN2-pTZSV28 was linearized with EcoRI (20 units for 1 h at 37°C), and its 3′ end was radiolabeled with [α-32P]dATP (25 pmol; specific activity, 800 Ci/mmol) and dATP, using the Klenow fragment [10 units for 1 h at 37°C (pH 7.5), 5 mM MgCl₂, 7.5 mM dithiothreitol]. This sample was deproteinized with phenol/chloroform, ethanol precipitated, resuspended in 20 μl of TE (pH 8.0), and subsequently cleaved with HindIII (20 units for 2 h at 37°C). C-pTZSV28 was treated similarly. Size markers were prepared by [γ-32P]-radiolabeling at the EcoRI site (as above) and by cleaving with either HindIII, MluI, SafI, or AccI to generate fragments with lengths of 58, 52, 45, and 33 base pairs, respectively. The samples and markers were electrophoresed on a 22% nondenaturing polyacrylamide gel for 3.5 h at 400 V, the gel was dried, and autoradiography was performed.

Quantitation of Inter-HN2-pTZSV28 and C-pTZSV28

For subsequent studies, it will be essential to add equal amounts of inter-HN2-pTZSV28 and C-pTZSV28 to cells in order to evaluate the relative ability of each to be replicated. Two methods were developed to establish relative yields of the two vectors.
RESULTS AND DISCUSSIONS

Duplex Oligonucleotide Containing a Nitrogen Mustard Interstrand Cross-Link. A partially duplex, dodecameric oligodeoxoribonucleotide, designated 1-M1-NH2-X1 (Fig. 1) was synthesized, characterized, and purified by us (30), and was incorporated into the human shuttle vector, pTZSV28 (Fig. 1). The attributes of 1-M1-HN2-X1 (Fig. 1) are that: (a) its duplex portion contains an AccI/SalI recognition site (underlined bases); (b) the two component oligonucleotides have overhangs that are complementary to the “sticky ends” generated by cleavage of DNA with the restriction endonucleases, MluI and XbaI, (top and bottom oligonucleotide, respectively, in Fig. 1); and (c) the cross-link is found in the target sequence, 5'-GAC-3', 3'-CTG-5'. The nitrogen mustard moiety is linked to the guanines in opposing strands at N(7)-positions and the guanine adducts are ring opened to their corresponding, formamidopyrimidine forms (30). There are two 5'-GAC-3', 3'-CTG-5' target sequences in the AccI/SalI restriction site, and 1-M1-HN2-X1 was shown (30) to have a cross-link in either one or the other of these targets; the rightmost sequence predominated (~80%).

Incorporation of 1-M1-HN2-X1 into pTZSV28. An SV40-based, human shuttle vector, pTZSV28 (Fig. 1), was constructed that was appropriate for the incorporation of 1-M1-HN2-X1 and subsequent biological studies (see “Materials and Methods”). pTZSV28 has a polylinker region characteristic of Messing vectors (Ref. 32; Fig. 1), except that a unique MluI restriction endonuclease recognition site was added between the HindIII and Spal sites for strategic reasons.

The incorporation of 1-M1-HN2-X1 into pTZSV28 used a 5-step procedure (see legend to Fig. 2), which ensured that one, and only one, copy of 1-M1-HN2-X1 was incorporated per genome. The ligation mixtures following step 5 of Fig. 2 were predominantly an equal mixture of closed and open circular DNA (Figs. 3 and 4). Closed circular forms of inter-HN2-pTZSV28 and C-pTZSV28 were isolated by cesium chloride density gradient centrifugation (Fig. 3). A simpler, 2-step protocol, whereby 1-M1-HN2-X1 was ligated into pTZSV28, which had been cleaved with MluI and XbaI, was judged inadequate, because the product could contain multiple, tandemly arrayed, copies of 1-M1-HN2-X1.

Characterization of the Nitrogen Mustard Interstrand Cross-Linked Shuttle Vector, Inter-HN2-pTZSV28. We wished to establish that inter-HN2-pTZSV28 had the properties expected of a vector containing an interstrand cross-link. The presence
Fig. 3. Purification of inter-HN2-pTZSV28 following ligation (step 5 of Fig. 2) by cesium chloride density gradient centrifugation ("Materials and Methods"). Fractions were collected from the bottom of the gradient, and an aliquot from each fraction (following ethanol precipitation) that contained 32P-radioactivity (located by Cerenkov counting) was electrophoresed through an agarose gel, which was subsequently dried and autoradiographed. Material migrating with a mobility characteristic of closed circular DNA appeared in fractions that eluted relatively early (e.g., Lanes 3), while material migrating with a mobility characteristic of linear and open circular DNA eluted relatively late (e.g., Lanes 8 and 9).

Fig. 4. Characterization of purified C-pTZSV28 and inter-HN2-pTZSV28. C-pTZSV28 following step 5 in Fig. 2 (Lane 1), as well as following its purification by cesium chloride density gradient centrifugation (Lane 2). Purified C-pTZSV28 treated with AccI (Lane 3) or PstI (Lane 4). Purified inter-HN2-pTZSV28 (Lane 5) was treated with AccI (Lane 6) or PstI (Lane 8). Purified inter-HN2-pTZSV28 and C-pTZSV28 were mixed and treated with AccI and the results (Lane 7) appear to approximate the sum of the results of AccI in Lanes 3 and 5.

Fig. 5. Demonstration that inter-HN2-pTZSV28 contains an interstrand cross-link. Purified C-pTZSV28 itself (Lane 1), and following denaturation/renaturation (Lane 2); the major band migrated with a mobility characteristic of single stranded pTZSV28. Purified inter-HN2-pTZSV28 itself (Lane 3), and following denaturation/renaturation (Lane 4); the major band migrated with a mobility characteristic of double-stranded pTZSV28. A mixture of inter-HN2-pTZSV28 and C-pTZSV28 (Lane 5) was denatured/renatured and gave results (Lane 6) that appeared to approximate the sum of the results in Lanes 2 and 4.

Fig. 6. Demonstration that inter-HN2-pTZSV28 contains a single cross-link. If inter-HN2-pTZSV28 contained a single 1-M1-HN2-X1 moiety per genome, then cleavage of its polylinker with EcoRI/HindIII would liberate the cross-link in a 45 base pair fragment (Fig. 1). Markers of 58, 52, 45, and 33 base pairs (Lanes 1 to 4, respectively) were generated as described in "Materials and Methods." Inter-HN2-pTZSV28 (Lane 5) and C-pTZSV28 (Lane 6) were digested with EcoRI. 32P-radiolabeled on their 3'-ends, and digested with HindIII ("Materials and Methods"). In each lane a band appears near the top of the autoradiogram, which represents the larger fragment containing 32P-radiolabel in its EcoRI site.

We wished to confirm that the procedure outlined in Fig. 2 did indeed yield a product with a single cross-link per genome. If inter-HN2-pTZSV28 had a single 1-M1-HN2-X1 moiety to subtle differences in conditions, the protocol was repeated with a mixture of inter-HN2-pTZSV28 and C-pTZSV28 and additive results were obtained (Fig. 5, Lane 6).

If inter-HN2-pTZSV28 contained a single cross-link, then cleavage of its polylinker with EcoRI/HindIII would liberate the cross-link in a 45-base pair fragment (Fig. 1). Markers of 58, 52, 45, and 33 base pairs (Lanes 1 to 4, respectively) were generated as described in "Materials and Methods." Inter-HN2-pTZSV28 (Lane 5) and C-pTZSV28 (Lane 6) were digested with EcoRI. 32P-radiolabeled on their 3'-ends, and digested with HindIII ("Materials and Methods"). In each lane a band appears near the top of the autoradiogram, which represents the larger fragment containing 32P-radiolabel in its EcoRI site.
incorporated between its MluI and XbaI sites, then its cleavage with HindIII and EcoRI should give a 45-base pair fragment containing the 1-M1-HN2-X1 moiety (Fig. 1); this expectation was observed experimentally (Fig. 6, Lane 5). In this experiment all contaminants of inter-HN2-pTZSV28, which did not have a 45-base pair poly linker, would have been detectable, because band intensity was derived principally from 32P-radio-labeled incorporated at the EcoRI end of the 45-base pair fragment and not from 1-M1-HN2-X1. No contaminants were apparent (Fig. 6).

Conclusions. We have developed a procedure (Fig. 2) to incorporate a single duplex, inter-strand cross-linked oligonucleotide into the human shuttle vector, pTZSV28, to give a product designated inter-HN2-pTZSV28. Furthermore, we have characterized inter-HN2-pTZSV28 and shown: (a) that it contains a chemical modified in its Accl/SalI site; (b) that this modification is likely to be an interstrand cross-link; and (c) that inter-HN2-pTZSV28 contains one and only one, cross-link per genome. Finally, a procedure was developed ("Materials and Methods") to quantitate the yield of inter-HN2-pTZSV28 versus C-pTZSV28, which will be of significance when both are placed into cells in order to determine their relative ability to be replicated.

Inter-HN2-pTZSV28 is being used to study the biological consequences of interstrand cross-links in both bacterial and human cells. The methods described herein and in our previous work (30) should be generally applicable and should permit the study of the biological consequence of interstrand cross-links derived from nitrogen mustard and other chemicals.

REFERENCES

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