Carcinogenic Chromium(VI) Induces Cross-Linking of Vitamin C to DNA in Vitro and in Human Lung A549 Cells†

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Received October 17, 2001; Revised Manuscript Received January 7, 2002

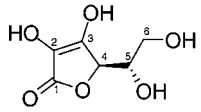
ABSTRACT: Reductive activation of carcinogenic Cr(VI) is required for the induction of DNA damage and mutations. Here, we examined the formation of Cr−DNA adducts in the reactions of Cr(VI) with its dominant biological reducer, vitamin C (ascorbate). Reductive conversion of Cr(VI) to Cr(III) by ascorbate produced stable Cr−DNA adducts, of which approximately 25% constituted ascorbate−Cr(III)−DNA cross-links. No evidence was found for the involvement of Cr(V) or Cr(IV) intermediates in the formation of either binary or ternary adducts. The cross-linking reaction was consistent with the attack of DNA by transient Cr(III)−ascorbate complexes. The yield of Cr(III)−DNA adducts was similar on dsDNA and AGT, ACT, or CT oligonucleotides and was strongly inhibited by Mg^{2+}, suggesting predominant coordination of Cr(III) to DNA phosphate oxygens. We also detected cross-linking of ascorbate to DNA in Cr(VI)-exposed human lung A549 cells that were preincubated with dehydroascorbic acid to create normal levels of intracellular ascorbate. Ascorbate−Cr−DNA cross-links accounted for approximately 6% of the total Cr−DNA adducts in A549 cells. Shuttle-vector experiments showed that ascorbate−Cr−DNA cross-links were mutagenic in human cells. Our results demonstrate that in addition to reduction of Cr(VI) to DNA-reactive Cr(III), vitamin C contributes to the genotoxicity of Cr(VI) via a direct chemical modification of DNA. The absence of Asc in A549 and other human cultured cells indicates that cells maintained under the usual in vitro conditions lack the most important reducing agent for Cr(VI) and would primarily display slow thiol-dependent activation of Cr(VI).

The carcinogenic potential of some chromium(VI) compounds has been well documented in epidemiological and animal studies (*1*−*3*). Mechanistic studies in vitro have shown that Cr(VI) is actually a procarcinogen that requires reductive activation to inflict DNA damage (*4*). Reduction of Cr(VI) in vivo is believed to be primarily nonenzymatic and driven by ascorbate (vitamin C) and nonprotein thiols, cysteine and glutathione (*5*−*7*). Several lines of evidence strongly suggest that for many cells in vivo ascorbate (Asc)[1] is the most important reducing agent for Cr(VI). Asc was identified chromatographically as the major reducing component in the rat lung (*8*), and the half-life of Cr(VI) in this tissue was found to be similar to that measured in buffer solutions with the equivalent concentrations of Asc (*6*). A strong inhibition of Cr(VI) reduction activity in ascorbate oxidase-treated tissue homogenates has further supported a major role for Asc in the metabolism of Cr(VI) in vivo (*9*, *10*). Kinetic studies have shown that reduction of Cr(VI) by Asc is a first-order reaction with respect to both reactants (*11*−*13*). The stoichiometry of the reduction reaction by Asc at neutral pH can be described as follows:

$$2\mathrm{Cr(VI)} + 3\mathrm{Asc}^- \rightarrow 2\mathrm{Cr(III)} + 3\mathrm{DHA} + 3\mathrm{H}^+$$

(DHA is dehydroascorbic acid)

The stoichiometry of the reaction implies that Asc acts as a two-electron donor. The absence of significant amounts of Cr(V) already in the presence of a 2-fold molar excess of the reducer (*14*−*16*) indicates that the first step in the reduction of Cr(VI) proceeds through the transfer of two electrons. This reaction produces Cr(IV) as the major intermediate form. The end product of the reduction is Cr(III) which forms stable coordinate complexes with two molecules of Asc (*17*).



ascorbic acid

It is generally believed that Cr(VI) initiates the carcinogenic process through the induction of mutations (*4*, *18*, *19*). Therefore, it is important to identify reduction pathways and DNA lesions that are responsible for the mutagenic activity of Cr(VI). Reductive conversion of Cr(VI) to Cr(III) by Asc in vitro has been previously found to result in Cr−DNA binding (*20*−*22*), Cr(III)-mediated interstrand DNA−DNA cross-links (*21*, *22*), and minor DNA breakage at high Cr(VI) concentrations (*15*, *23*). Reduction of Cr(VI) by ascorbate did not lead to a detectable production of oxidized DNA bases (*24*). DNA cross-links are believed to be the major

† This work was supported by Grant ES 08786 from the National Institute of Environmental Health Sciences.

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[1] Abbreviations: Asc, ascorbic acid; DHA, dehydroascorbic acid; DDB, 1,2-diamino-4,5-dimethoxybenzene dihydrochloride; OPDA, *o*-phenylenediamine dihydrochloride.

10.1021/bi011942z CCC: $22.00
Published on Web 02/08/2002

form of the polymerase-blocking lesions produced in these reactions (*22*), which points to their potentially important role in the induction of cytotoxicity but not mutagenesis. A direct comparison of the mutagenic potency of different Cr(III)−DNA adducts (*25*) and analysis of mutagenic responses produced by reductive conversion of Cr(VI) by cysteine (*26*) showed the high mutagenic potential of ternary Cr−DNA adducts. Small binary Cr(III)−DNA adducts were only weakly mutagenic following replication of the pSP189 plasmid in human cells (*25*). The metabolism of Cr(VI) by biological thiols produces ternary glutathione−Cr(III)−DNA and cysteine−Cr(III)−DNA adducts (*27*). The yield of each ternary adduct appeared to be directly linked to the intracellular concentration of the specific thiol and its reducing activity (*27*, *28*), suggesting that participation in the Cr(VI) reduction might be important for the subsequent cross-linking reaction. Given a high mutagenic potential of ternary adducts and a major role of Asc in Cr(VI) metabolism, it is important to determine whether this type of DNA adduct can be formed in the reactions with Asc. The inability of Asc-based reactions to produce ternary adducts along with a low yield of interstrand DNA cross-links at the biologically relevant concentrations of the reactants (*21*) could mean that, in contrast to thiols, the Asc-dependent pathway is relatively nongenotoxic. The low levels of major genotoxic lesions in the reactions with this two-electron reducer could also be indicative of the importance of Cr(V) in the formation of the most significant forms of DNA damage. In contrast to intracellular reactions, extracellular reduction of Cr(VI) by Asc represents a well-understood detoxification process that yields nontoxic, impermeable Cr(III) complexes (*6*, *18*).

In this work, we examined the potential formation of Asc−DNA cross-links in reactions with Cr(VI) in vitro and in human lung A549 cells. Cr(III)-mediated cross-linking of Asc to DNA was detected in both experimental systems. The formation of both binary and ternary adducts on oligonucleotides of base-specific composition was consistent with coordination of Cr(III) to DNA phosphates. The highest yield of Asc−DNA cross-links was found in the reactions with a large molar excess of Asc over Cr(VI), indicating that Cr(V) is unlikely to play a significant role in the formation of these adducts. Shuttle-vector experiments showed that ascorbate−Cr−DNA cross-links were mutagenic in human cells. Our results demonstrate that Asc contributes to the genotoxicity of Cr(VI) through the production of reactive Cr(III) and ternary DNA adducts. To our knowledge, this is the first example of direct chemical modification of DNA by vitamin C.

EXPERIMENTAL PROCEDURES

Materials. L-Ascorbic acid (ACS reagent), *o*-phenylenediamine dihydrochloride (OPDA), and all HPLC solvents and reagents for electrophoresis were from Sigma (St. Louis, MO). Asc and all buffer solutions were passed through Chelex-100 columns to remove residual redox-active metals (*29*). Purified solutions of Asc were kept on ice and used within 30 min of purification. Chelex-100 resin (200 mesh) and Bio-Gel P-30/P-6 columns were purchased from Bio-Rad (Hercules, CA). Na_2CrO_4 (ACS reagent) and $[Cr(H_2O)_4Cl_2]Cl{\cdot}2H_2O$ (99.995% pure) were from Aldrich (Milwaukee, WI). 1,2-Diamino-4,5-dimethoxybenzene dihydrochloride (DDB) was supplied by Molecular Probes (Eugene, OR). [^{51}Cr]Chromate, [^{51}Cr]chromium chloride, and ^{14}C-labeled ascorbic acid were from Amersham (Arlington Heights, IL). The pSP189 plasmid (M. Seidman) was purified using a kit from Qiagen. Commercial calf thymus DNA (Sigma) was additionally purified via the proteinase K/phenol procedure (*30*). Oligonucleotides were synthesized by Gibco BRL: AGT oligo (39 nucleotides), 5′-AATGGTTGGTAGATA-GATGTTGAATGTTAGTAGAGGTAA; ACT oligo (39 nucleotides), AATCCTTCCTACATACATCTTCAATCT-TACTACACCTAA; and TC oligo (40 nucleotides), CTTC-TCCCTCTCTCCTTCTCTCTTTCCTCCCTCTTTTCCT.

Cells. Human lung A549 cells were obtained from American Tissue Culture Collection and grown at 37 °C in an atmosphere containing 95% air and 5% CO_2. A549 cells were propagated in 90% F-12K medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gemini Bio-Products) and a 1% penicillin/streptomycin mixture. Cells were treated at approximately 80% confluence in 100 mm dishes.

Formation of Cr−DNA Adducts in the Reactions with Cr(VI). A standard reaction mixture (final volume of 50 μL) contained 25 mM MOPS buffer (pH 7.0), 1 mM Asc, 2 μg of pSP189 DNA, and various concentrations of Na_2CrO_4. A set of Cr(VI) treatments of DNA was also performed in 25 mM sodium phosphate buffer (pH 7.0) or in 25 mM MOPS buffer containing 5 mM EDTA or 10 mM $MgCl_2$. Mg ions were preincubated with DNA for 10 min prior to the start of the reduction with Asc. Samples were incubated at 37 °C for 30 min, and unbound Cr and Asc were removed by size-exclusion chromatography on Bio-Gel P-30 columns. DNA was precipitated overnight with 2 volumes of cold 100% ethanol and 200 mM NaCl. DNA precipitates were collected by centrifugation (10000*g* for 10 min at 4 °C) and washed twice with 70% ethanol. The ethanol precipitation procedure removed all Cr(III) complexes that were ionically associated with DNA (15−25% of the initially bound Cr). Use of higher concentrations of NaCl (up to 1 M) did not result in the additional release of Cr(III) from DNA. The number of Cr−DNA adducts was determined using radiolabeled [^{51}Cr]-chromate (*29*).

Assessment of Asc−DNA Binding in Vitro. DNA or oligonucleotides were treated with Cr(VI) and/or Asc and purified as described above. DNA pellets were dissolved in 50 mM phosphate buffer (pH 3.0) containing 5 mM DPTA and 0.9 mM *o*-phenylenediamine (OPDA). Samples were incubated in the dark for 24 h at 4 °C. Released Asc was oxidized by the addition of 0.5 unit of ascorbate oxidase and incubation at 37 °C for 30 min. OPDA reacts with DHA to produce a fluorescent quinoxaline derivative (*31*). The amount of DHA−OPDA conjugates was determined by HPLC with fluorescent detection (excitation at 355 nm, emission at 425 nm). Chromatographic separation was performed on an Ultrasphere ODS 5 μm, 4.6 mm × 250 mm column using isocratic elution with 50 mM phosphate buffer and 20% acetonitrile (pH 7.8) and a flow rate of 1 mL/min. The presence of OPDA during the release phase minimized losses of Asc, presumably by forming a stable adduct with DHA and preventing additional rearrangements. In the initial experiments, we also measured the amount of DNA-bound Asc by including ^{14}C-labeled ascorbate in the Cr(VI) reduction mixture. We found that more than 90% of the [^{14}C]Asc was dissociated from DNA during incubation

in the phosphate/DPTA buffer. However, radiolabeled Asc was not routinely used for quantitation of Asc−DNA cross-links due to a very high batch-to-batch variability in the quality of the isotope samples and the presence of impurities.

To examine the possibility of Asc cross-linking to Cr(III)−DNA adducts, pSP189 DNA was treated with 20, 40, or 60 μM $[Cr(H_2O)_4Cl_2]Cl\cdot 2H_2O$ for 30 min at 37 °C in 25 mM MES (pH 6.0). DNA was passed through P-30 columns and precipitated with ethanol as described above. Cr(III)-modified DNA was reacted with 1 mM Asc for 2 h at 37 °C in either 25 mM MOPS (pH 7.0) or 25 mM MES (pH 6.0). Unreacted Asc was removed via the P-30/ethanol procedure. The amount of DNA-bound Asc was determined by HPLC as described above. Cr−DNA binding was quantified by inclusion of trace amounts of ^{51}Cr-labeled chromium chloride in a parallel set of samples (*20*).

Formation of Ternary DNA Adducts in the Reactions with Cr(III)−(Ascorbate)$_2$ Complexes. The dark green $Cr(Asc)_2(H_2O)_7$ complex was synthesized according to a published procedure (*17*). Purified Cr(III)−$(Asc)_2$ complexes were reacted with 2 μg of pSP189 DNA at 37 °C in 25 mM MOPS (pH 7.0) or 25 mM MES (pH 6.0) for 2 h at 37 °C. DNA was purified by P-30 chromatography and ethanol precipitation as described above. The number of Asc−DNA cross-links was calculated from the amount of Asc released from DNA after incubation for 24 h in 50 mM phosphate/5 mM DPTA buffer (pH 3.0) at 4 °C. The amount of Asc was determined by HPLC analysis of the fluorescent DHA−OPDA derivative as described above.

Determination of the Amount of Intracellular Ascorbate. A549 cells had undetectable intracellular vitamin C (<0.5 μM) when cultured under standard conditions. To deliver Asc into the cytoplasm, cells were incubated with either the reduced or oxidized form of vitamin C. Pretreatment of cells with the reduced form of vitamin C (Asc) was carried out in complete medium for 3 or 24 h. Incubation of cells with various concentrations of DHA (oxidized vitamin C) was performed in a Krebs buffer supplemented with 0.5 mM D-glucose. After incubations with Asc or DHA, dishes were washed with PBS and cells were collected by trypsinization. After one more wash in PBS (1100*g* for 5 min at 4 °C), cell pellets were resuspended in 50 μL of a PBS/5 mM DPTA solution followed by the addition of 200 μL of 50 mM methanesulfonic acid, and then samples were twice frozen (−70 °C) and thawed (37 °C). These conditions did not lead to any appreciable loss of Asc as determined in the recovery experiments with spiked samples. Asc-containing supernatants were obtained after centrifugation at 12000*g* for 10 min at 4 °C. Aliquots of cellular extracts (50 μL) were mixed with 0.5 M sodium acetate (pH 6.0), 0.5 unit of ascorbate oxidase, and 0.9 mM OPDA in a total reaction volume of 250 μL. The derivatization reaction was performed at 37 °C for 30 min, and fluorescent DHA−OPDA conjugates were quantified by HPLC as described above.

Determination of the Number of Ascorbate Cross-Links in Cellular DNA. A549 cells were incubated for 90 min at 37 °C in Krebs buffer supplemented with 0.5 mM D-glucose and 2 mM DHA. DHA-containing medium was removed, and cells were washed with PBS and then exposed to Cr(VI) in serum-free medium for 3 h. Cells were washed in PBS, collected by trypsinization, and then spun down (1100*g* for 5 min). After an additional wash with PBS, cell pellets were resuspended in 50 μL of 25 mM MOPS (pH 7.0) followed by addition of 0.5 mL of a lysis solution containing 25 mM MOPS (pH 7.0), 0.5% Triton X-100, and 0.1 mg/mL RNase A. After incubation for 30 min at 37 °C, samples were supplemented with 0.5% SDS, sheared five times with a 25 gauge needle, and digested with 1 mg/mL proteinase K (60 min at 37 °C). DNA was extracted twice with phenol and once with chloroform and precipitated with 2 volumes of cold ethanol overnight. Agarose electrophoresis showed that isolated DNA had a high molecular weight and was free of visible RNA contamination. DNA precipitates were washed twice with 70% ethanol, and DNA-bound Asc was released by incubation in 50 mM sodium phosphate and 5 mM DPTA (pH 3.0) for 24 h at 4 °C. Ascorbate oxidase (0.5 unit) and 0.1 mM 1,2-diamino-4,5-dimethoxybenzene (DDB) were added to each sample followed by incubation at 37 °C for 30 min. DHA forms a highly fluorescent conjugate with DDB (*32*), which was separated and quantified by HPLC using a 4.6 mm × 250 mm Ultrasphere ODS 5 μm column. Solvent A was 50 mM phosphate (pH 2.0) and solvent B acetonitrile. Stepwise gradient elution was used as follows: 15% B for 8 min, 50% B for 13 min, and 15% B for 9 min. The flow rate was 1 mL/min. The fluorescence detector was set to 371 nm (excitation) and 458 nm (emission). All DDB-based measurements were corrected for the loss of Asc occurring during phosphate release.

Shuttle-Vector Experiments. Ascorbate−Cr(III)−DNA cross-links (5.2 Asc molecules/1000 bp of DNA) were formed by reacting 0.5 mM Cr(III)−$(Asc)_2$ complex with pSP189 DNA in 25 mM MES buffer as described above. Control reactions for examining the potential formation of nonspecific DNA damage were performed in 25 mM phosphate. The presence of phosphate completely blocked the generation of Cr−DNA adducts. A set of control and cross-link-containing DNA samples was also incubated in the presence of 50 mM sodium phosphate (pH 7.0) for 24 h at 4 °C, which caused dissociation of approximately 90% of the ternary adducts. Shuttle-vector experiments were performed as described previously (*26*). In brief, control and cross-link-containing plasmids were transfected into human fibroblasts and allowed to replicate for 48 h. Replicated progeny of plasmids was isolated and then electrotransfected into an indicator MBL50 *Escherichia coli* strain to score mutations in the *supF* gene. The mutation frequency was calculated by dividing the number of arabinose/ampicillin-resistant colonies by the number of ampicillin-resistant colonies.

Other Procedures. The kinetics of chromate reduction and electrophoretic analysis of Cr-modified DNA were assessed as previously described (*26*). The amount of DNA-bound Cr was determined by graphite furnace atomic absorption spectroscopy using Zeeman background correction (Perkin-Elmer GF-AAS, model 41002L). The wavelengh was 357.5 nm with a bandwidth of 0.7 nm. A five-step graphite furnace program was used: (1) 110 °C, ramp for 1 s, hold for 20 s; (2) 130 °C, ramp for 5 s, hold for 30 s; (3) 1500 °C, ramp for 10 s, hold for 20 s; (4) 2300 °C, hold for 5 s; and (5) 2500 °C, ramp for 1 s, hold for 2 s.

RESULTS

Formation of Stable Cr−DNA Adducts in the Reaction of Cr(VI) with Ascorbate. Cr(III) complexes can bind to DNA

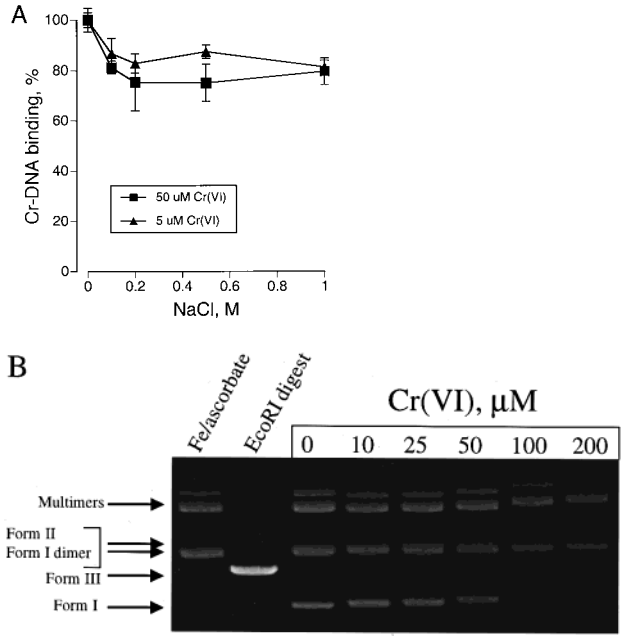


FIGURE 1: Formation of stable Cr−DNA adducts in the reaction of Cr(VI) with 1 mM ascorbate. Reactions were performed at 37 °C for 30 min in 25 mM MOPS (pH 7.0). (A) Stability of Cr−DNA binding in the presence of NaCl. DNA was treated with 5 or 50 μM chromate and 1 mM Asc followed by the removal of unbound reactants by P-30 chromatography. Purified DNA was incubated with 0−1 M NaCl for 20 min and then passed through P-30 columns again. Shown are means ± the standard deviation (SD) of three independent determinations. (B) Agarose electrophoresis of Cr(VI)-treated pSP189 DNA. Each sample contained 1 mM Asc, 0.4 μg of plasmid DNA, and various amounts of Na_2CrO_4 in a volume of 25 μL. Samples were incubated for 30 min followed by immediate loading onto 1% agarose gels. Fe/ascorbate-DNA was treated with 10 μM $FeCl_3$ and 500 μM Asc to produce nicked plasmids: form I, intact supercoiled plasmids; form II, open circular plasmids containing one or more single-strand breaks; and form III, linearized plasmids.

either in the inner sphere mode (stable adducts) or through outer sphere coordination (ionic binding). In reactions of Cr(VI) with cysteine, approximately 40−60% of the initially bound Cr(III) complexes constituted the ionically bound fraction (*29*). Rapid formation of Cr(III)−hydroxo species at neutral pH can potentially lead to an even greater percentage of ionic binding. Figure 1A shows that in the presence of elevated concentrations of NaCl 15−25% of the initially bound Cr could be removed from DNA, which represents ionically associated complexes. The presence of 200 mM NaCl was sufficient to dissociate all ionic complexes since there was no additional release of Cr from DNA at higher ionic strengths. In all subsequent DNA binding experiments, we removed ionically bound Cr using precipitation of DNA with ethanol and 200 mM NaCl.

The presence of stable coordinate complexes between Cr(III) and DNA was further confirmed by agarose electrophoresis of plasmid DNA that had been treated with Cr(VI) and 1 mM Asc (Figure 1B). The reduced mobility of the lower DNA band and a diminished staining with ethidium bromide reflect unwinding of supercoiled molecules and the potential presence of structural distortions in Cr-modified DNA duplexes, respectively (*26*). All these changes in plasmid DNA can only be caused by stable Cr(III)−DNA adducts, whereas ionic Cr(III) complexes would be sequestered by a strong Cr(III) chelator (EDTA) that was present in the electrophoretic buffer at a concentration of 1 mM. Notably, there was no detectable decrease in the relative intensity of the supercoiled DNA monomers (form I monomer), suggesting the absence of significant DNA breakage during reduction of 0−200 μM Cr(VI) with 1 mM Asc. Similar results were also found in the experiments with ΦX174 DNA preparations that contained only relaxed and supercoiled monomers (not shown).

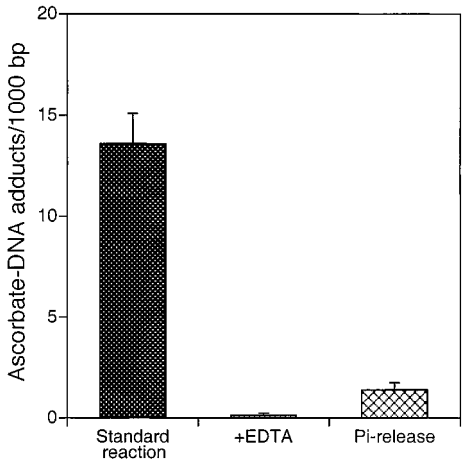


FIGURE 2: Binding of [^{14}C]ascorbate to DNA in the presence of Cr(VI). The standard reaction mixture contained 25 mM MOPS (pH 7.0), 100 μM Cr(VI), 1 mM [^{14}C]ascorbate, and 2 μg of pSP189 DNA. After incubation for 30 min at 37 °C, DNA was purified by the P-30/ethanol procedure. For the +EDTA data, the reaction mixture also contained 5 mM EDTA. For the Pi-release data, Cr-modified DNA was incubated in a 50 mM phosphate (pH 3.0)/5 mM DPTA solution for 24 h at 4 °C and then passed through P-30 columns. Means ± SD from at least three independent determinations.

Cross-Linking of Ascorbate to DNA. ^{14}C-labeled Asc was used in the initial experiments to determine whether there was any stable binding of vitamin C to DNA following Cr(VI) reduction. Although the results were always positive, highly variable amounts of DNA-bound radioactivity were detected in different batches of samples. We found that the presence of insoluble and soluble impurities in the radiolabeled preparations of Asc was responsible for this inconsistency in DNA binding experiments. In all subsequent experiments, ^{14}C preparations were analyzed by HPLC to determine the exact amount of [^{14}C]Asc and its specific activity. The insoluble material was typically removed by centrifugation at 20000*g* for 20 min at 4 °C. The experiments with purified [^{14}C]Asc were more reproducible as indicated by the relatively small error bars for each set of samples (Figure 2). Cross-linking of Asc to DNA was completely blocked in the presence of the Cr(III) chelator (EDTA). The addition of EDTA also abolished the formation of Cr−DNA adducts in the 0−200 μM range of Cr(VI) concentrations. The majority of the Cr(III)−DNA adducts can be disrupted during overnight incubation in the presence of inorganic phosphate (*26*). When Asc- and/or Cr-adducted DNA was incubated in the presence of inorganic phosphate, a loss of approximately 90% of the initially bound Asc was observed (Figure 3). Collectively, these experiments indicated that Cr(III) was responsible for the cross-linking of Asc to DNA.

The ability of phosphate to dissociate Asc−DNA cross-links was further explored for the development of an HPLC

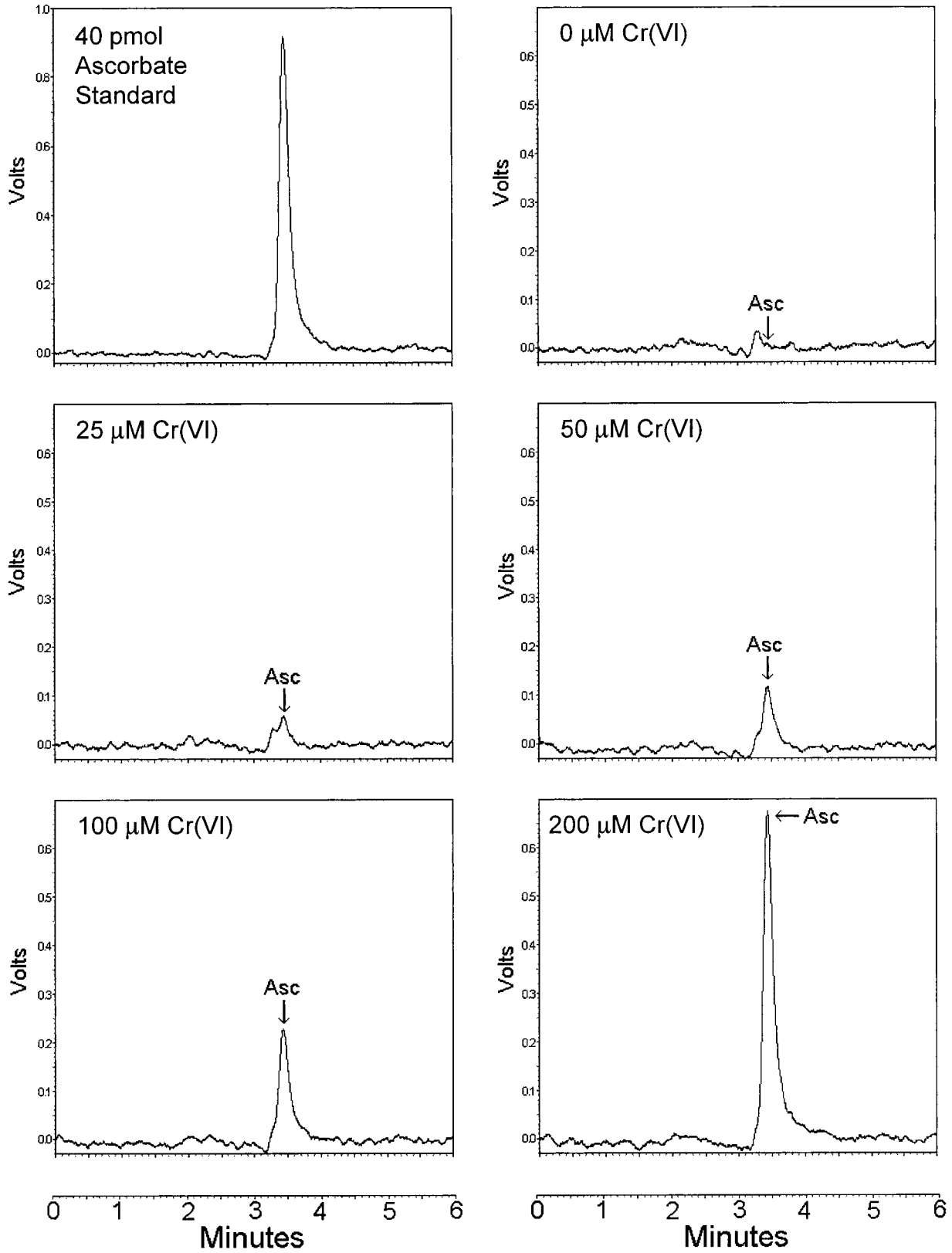


FIGURE 3: Determination of the amount of ascorbate−DNA cross-links by HPLC. Asc−DNA cross-linking was performed in 25 mM MOPS (pH 7.0) as described in the legend of Figure 2 except that nonradioactive Asc was used. Cr-modified DNA was incubated in the phosphate/DPTA buffer; released Asc was converted into a fluorescent DHA−OPDA derivative and detected by HPLC as described in Experimental Procedures.

assay based on measurement of the amount of released Asc. The oxidized form of Asc, DHA, reacts rapidly with OPDA to form a fluorescent quinoxaline derivative (*31*) that can be identified by HPLC. The conversion of Asc to DHA can be readily accomplished by the addition of ascorbate oxidase. Figure 3 shows typical HPLC profiles obtained from control and Cr(VI)- and/or Asc-treated DNA samples. There was no detectable Asc peak in DNA samples treated with Asc alone, whereas increasing concentrations of Cr(VI) led to progressively higher peaks of the DHA−OPDA derivative.

The detection limit for this assay is 1 Asc molecule/10000 bp for 2 μg DNA samples, which is several times higher than the sensitivity of the radioactivity procedure. The entire HPLC run was only 6 min, which allowed rapid processing of a large number of samples. The measurements of the amount of DNA-bound Asc by the HPLC assay or using the radioactive method were very close. For example, modification of DNA with 100 μM Cr(VI) was found to result in cross-linking of 13.3 ± 1.5 Asc molecules/1000 bp ([^{14}C]Asc assay) versus 14.5 ± 3.1 Asc molecules/1000 bp (HPLC). The number of Asc−DNA cross-links increased linearly as a function of Cr(VI) concentration when the reaction mixtures contained 1 or 2 mM Asc (Figure 4A). Using a more sensitive DHA−DDB HPLC assay, dose-dependent formation of Asc−DNA cross-links was also observed in a low micromolar Cr(VI) concentration range (Figure 4A, inset). In the reactions with 0.2 mM Asc, the yield of cross-links increased linearly up to 50 μM Cr(VI) and then plateaued. This nonlinearity most likely reflects the fact that reduction of 100 and 200 μM Cr(VI) would consume all or almost all the Asc, and therefore, only the initially produced Cr(III) would be able to react with Asc to form DNA-reactive complexes. A 10-fold variation in the concentration of Asc (0.2−2 mM) did not significantly affect the number of ascorbate−DNA adducts as long as the reducer was present in a molar excess of at least 4-fold. However, the reduction reactions with the physiological Asc concentration (1 mM) produced a higher relative yield of Asc−DNA adducts than 0.2 mM Asc reactions (Figure 4B). Even in the range of 25−50 μM Cr(VI) where Asc was present in excess in both reaction mixtures, 1 mM Asc samples contained a significantly higher fraction of Asc−DNA cross-links (25.9 ± 1.1 vs 14.9 ± 0.2% for 1 and 0.2 mM Asc reactions, respectively). A relative yield of Asc−DNA cross-links in 1 mM Asc reactions remained almost the same when the Asc:Cr(VI) ratio was varied from 200:1 to 10:1 (5−100 μM Cr). Lowering the Asc:Cr ratio to 5:1 (1 mM Asc and 200 μM Cr) led to a significantly diminished formation of ternary adducts (15.5% yield). A decreased percentage of Asc−DNA adducts in 0.2 mM Asc reactions even at low Cr(VI) concentrations primarily reflected a higher total level of Cr−DNA binding in comparison to that in 1 mM Asc-based samples (Figure 4C). A relatively lower yield of total Cr−DNA adducts in 0.2 mM Asc and 200 μM Cr(VI) samples probably resulted from the incomplete reduction of Cr(VI) by the equimolar concentration of Asc since complete conversion to Cr(III) would require a 1.5-fold molar excess of Asc (*11*−*13*).

Mechanism of Ascorbate−DNA Cross-Linking. Reduction of Cr(VI) in the presence of 1 mM Asc was very fast with a $t_{1/2}$ of 0.9 min, and more than 95% of the Cr(VI) was reduced during the first 5 min of the reaction (Figure 5A). In all previous experiments, we measured the amount of Asc−DNA adducts following 30 min incubations which included both the actual reduction period and a significantly longer postreduction interval. To determine which period of time was important for the formation of ternary adducts, we measured the number of Asc−DNA cross-links formed during the first 5 min of reduction and during the postreduction interval from 5 to 30 min (Figure 5B). The results clearly showed that a short period of Cr(VI) reduction was critically important for efficient Asc−DNA cross-linking

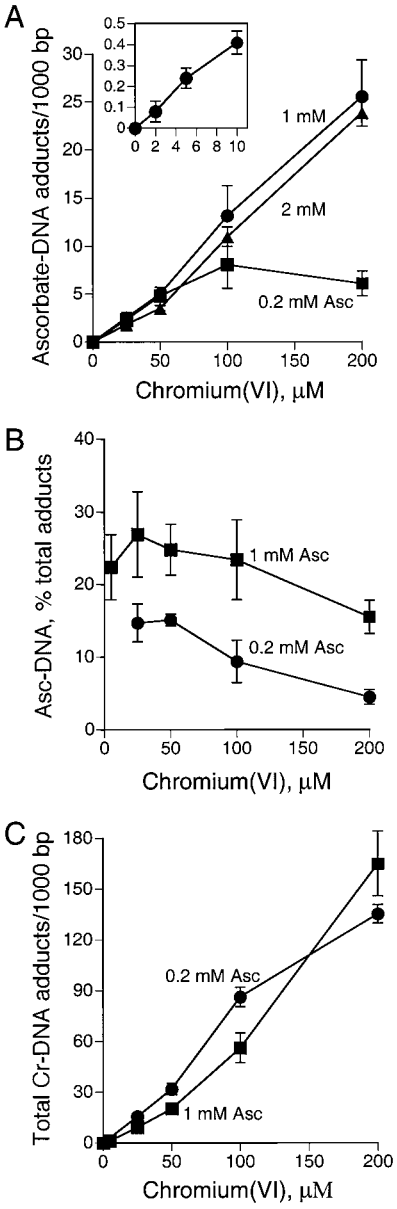


FIGURE 4: Dose dependence and relative yield of ascorbate−DNA adducts. Each reaction mixture contained 25 mM MOPS (pH 7.0), 2 μg of pSP189 DNA, and the indicated concentrations of Asc and Cr(VI). The ratios of Asc to Cr(VI) were 80:1, 40:1, 20:1, and 10:1 for 2 mM Asc reactions, 40:1, 20:1, 10:1, and 5:1 for 1 mM Asc reactions, and 8:1, 4:1, 2:1, and 1:1 for 0.2 mM Asc reactions. The amount of DNA-bound Asc in reaction mixtures containing 25−100 μM Cr(VI) was determined by the OPDA HPLC assay. The number of Asc−DNA cross-links in samples containing 1 mM Asc and 2, 5, or 10 μM Cr(VI) (Asc:Cr ratio of 500:1, 200:1, or 100:1, respectively) was quantified by the DDB HPLC procedure. The level of total Cr−DNA binding was determined using [^{51}Cr]-chromate. Shown are means ± SD of three to six independent determinations. If not seen, error bars were smaller than the symbols. (A) Formation of Asc−DNA cross-links as a function of Cr(VI) and Asc concentrations. The inset shows the yield of Asc−DNA cross-links in the reaction mixtures containing 1 mM Asc and 0−10 μM Cr(VI). (B) Relative yield of ascorbate−DNA cross-links. (C) Total Cr−DNA binding in the reaction mixtures containing 0.2 and 1 mM Asc.

since the yield of ternary adducts was essentially identical for both the 0−30 and 0−5 min intervals, and the post-reduction period (5−30 min) produced only a small amount of cross-links (15−20% relative to the 0−30 min samples). This rapid decrease in the level of formation of Asc−DNA

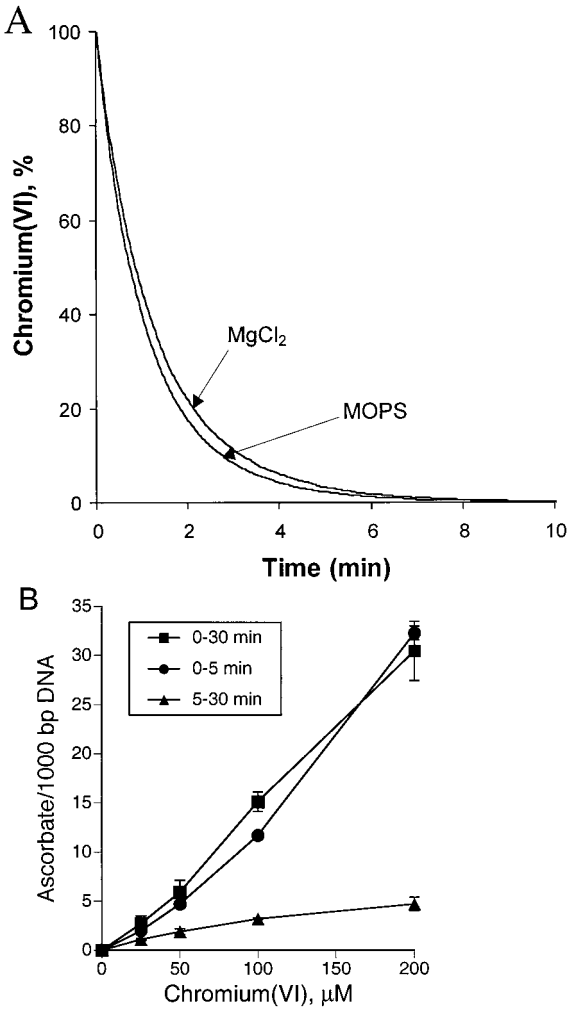


FIGURE 5: Ascorbate−DNA cross-linking during different phases of Cr(VI) reduction. Each reaction mixture contained 25 mM MOPS (pH 7.0), 2 μg of plasmid DNA, 1 mM Asc, and the indicated concentrations of Cr(VI). Some samples were preincubated with 10 mM $MgCl_2$ for 10 min prior to the start of the reaction (addition of Asc). Samples were incubated at 37 °C for the indicated periods of time, and DNA was purified by the P-30/ethanol procedure. The amount of DNA-bound Asc was determined by the OPDA HPLC assay. (A) Reduction kinetics of Cr(VI). For the MOPS trace, standard reaction mixture containing 50 μM Cr(VI), 1 mM Asc, and 25 mM MOPS (pH 7.0). For the $MgCl_2$ trace, reaction mixtures were supplemented with 10 mM $MgCl_2$. Reduction was followed by the decrease in chromate absorbance at 372 nm. (B) Formation of ascorbate−DNA cross-links: 0−30 min, complete reaction; 0−5 min, samples passed through P-30 columns after incubation for 5 min; and 5−30 min, DNA was added to the reaction mixture 5 min after the start of Cr(VI) reduction. Shown are means ± SD of four to eight independent determinations. If not seen, error bars were smaller than the symbols.

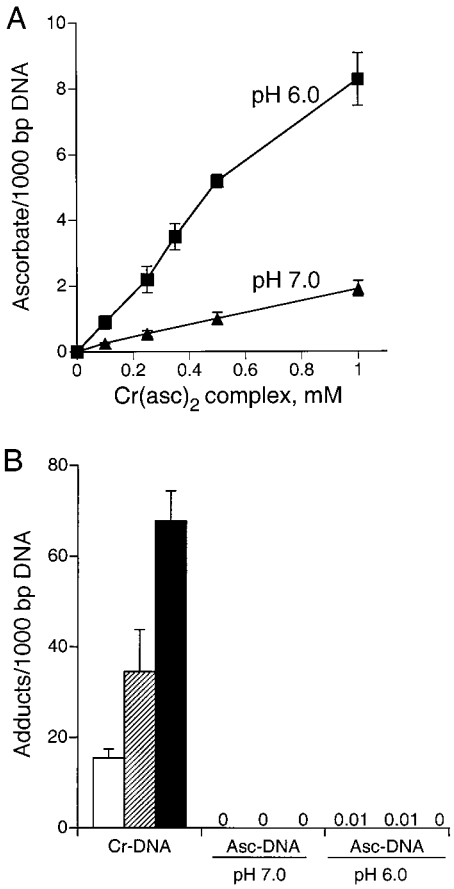


FIGURE 6: Formation of ascorbate−DNA adducts in reactions with Cr(III)−$(Asc)_2$ complexes or Cr(III)-modified DNA. Samples containing 25 mM buffer, 2 μg of pSP189 DNA, and the indicated concentrations of the Cr(III)−$(Asc)_2$ complex were incubated for 2 h at 37 °C. DNA was purified by the P-30/ethanol procedure. The number of ascorbate−DNA cross-links was determined by the OPDA HPLC assay. Shown are means ± SD of three to six independent determinations. (A) Formation of ternary adducts in the reaction with the Cr(III)−$(Asc)_2$ complex. Reactions were performed in 25 mM MOPS (pH 7.0) or 25 mM MES (pH 6.0). (B) Lack of ascorbate cross-linking to Cr(III)-modified DNA. DNA was treated with 20, 40, or 60 μM $[Cr(H_2O)_4Cl_2]Cl{\cdot}2H_2O$, purified, and then incubated with 1 mM Asc in 25 mM MOPS (pH 7.0) or 25 mM MES (pH 6.0).

adducts immediately after the completion of Cr(VI) reduction could reflect a very fast conjugation of reactive Cr(III)−Asc complexes with another Asc molecule, producing much less reactive Cr(III)−$(Asc)_2$ complex. To investigate this possibility, we isolated the Cr(III)−$(Asc)_2$ complex from Asc/Cr(VI) reactions (*17*) and then reacted it with DNA (Figure 6A). Although cross-linking of Asc to DNA was observed under these conditions, a normalized yield of ternary adducts at pH 7.0 was ~75.7 times lower than that from Cr(VI)-based reactions (0−30 min samples). The level of formation of Asc−DNA cross-links at pH 6.0 was 4.5 times higher than at neutral pH, but it was still 17 times lower than in Cr(VI) reactions. These results confirmed a very weak reactivity of Cr(III) complexes coordinated with two Asc molecules. It is likely that the residual cross-linking activity detected in the postreduction Cr(VI) samples (5−30 min) largely reflected the presence of a small amount of Cr(III) complexes containing one Asc ligand.

We next examined the possibility whether DNA-bound Cr(III) could capture Asc from the reaction mixture. Supercoiled pSP189 DNA was treated with 20, 40, and 60 μM $[Cr(H_2O)_4Cl_2]Cl{\cdot}2H_2O$ that produces $[Cr(H_2O)_4Cl_2]^+$ cation upon dissolution in aqueous medium. Two Cl ligands can be readily displaced by H_2O or in the reaction with DNA. Cr−DNA binding was performed at pH 6.0, which avoided the formation of poorly soluble Cr(III)−hydroxo species (*20*). Figure 6B shows that Asc was not cross-linked to Cr(III)-modified DNA during a 2 h incubation at either pH 7.0 or 6.0. The absence of ternary adducts even at pH 6.0 is important because the reactivity of Cr(III) at this pH is up to 10 times higher than that in neutral solutions (*29*).

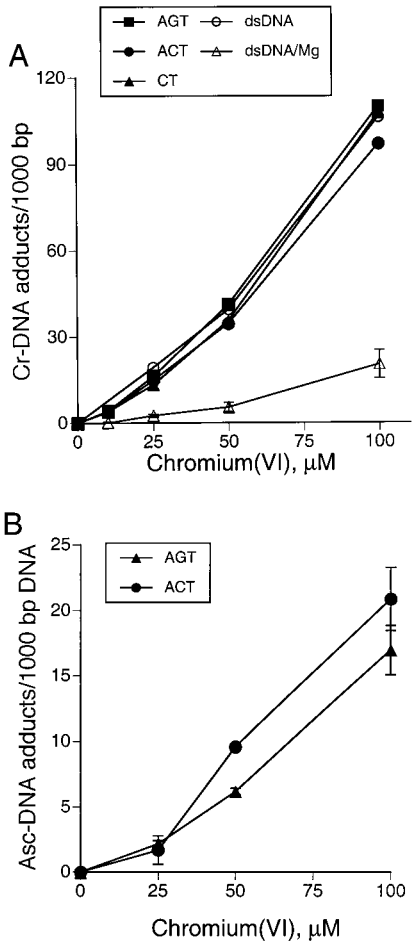


FIGURE 7: Lack of base specificity in the formation of Cr−DNA adducts. Each reaction mixture contained 25 mM MOPS (pH 7.0), 1 mM Asc, 2 μg of DNA, and the indicated concentrations of Cr(VI). The formation of Cr adducts was assessed using AGT-, ACT-, or CT-containing single-stranded oligonucleotides and double-stranded calf thymus DNA. Samples were incubated for 30 min at 37 °C followed by purification of DNA by the P-30/ethnol procedure. The levels of Cr−DNA and Asc−DNA adducts were determined using ^{51}Cr(VI) and the OPDA HPLC assay, respectively. (A) Cr−DNA binding. For dsDNA/Mg data, reaction mixtures contained 10 mM $MgCl_2$. Shown are means from six measurements. Excluding Mg-containing samples, SD values were not shown for clarity. (B) Ascorbate−DNA cross-linking. Shown are means ± SD from three experiments.

To determine whether there was any nucleotide preference in the formation of Cr−DNA adducts, we assessed Cr and Asc binding to single-stranded oligonucleotides of base-specific composition (Figure 7A,B). Many metals show strong binding to N7-G which is the most nucleophilic site in DNA (*33*). Pyrimidine bases typically do not exhibit significant affinity for metals due to the absence of strong nucleophilic groups. If Cr(III) preferentially binds to G, a higher level of binding to the AGT oligo relative to the ACT or TC oligo should be observed. Figure 7A shows that the formation of total Cr−DNA adducts was very similar for all three oligonucleotides over the entire range of Cr(VI) concentrations. The yield of Cr adducts in the samples with double-stranded DNA was essentially identical to that with the single-stranded oligos. We also found only a marginal difference in Cr binding to linear and supercoiled DNA in the reactions with 25, 50, and 100 μM Cr(VI) (78.4, 86.9, and 83.3% relative binding for supercoiled DNA, respectively). Collectively, these results are consistent with Cr binding to the DNA−phosphate backbone. Mg^{2+} ions bind almost exclusively to the DNA phosphates (*34*), and one would expect to observe a strong inhibitory effect on the number of Cr−DNA adducts in the presence of this metal. We found that the addition of Mg^{2+} ions completely blocked Cr−DNA binding at the lowest Cr(VI) concentration, whereas the number of adducts in the higher range was 5−8 times lower than in control reactions. Importantly, this inhibitory effect on Cr−DNA binding was not associated with significant changes in the reduction kinetics in the presence of Mg^{2+} (Figure 5A). Incomplete inhibition of Cr−DNA binding was probably caused by a partial sequestration of Mg^{2+} by Asc (*35*). A rapid reduction of Cr(VI) by 1 mM Asc produces large amounts of Cr(III) over a very short period of time, which should lead to a more effective competition with Mg^{2+} for the binding sites on DNA. The reduction of Cr(VI) in the presence of 25 mM sodium phosphate abolished the formation of Cr−DNA adducts (not shown). Similarly to the results for the total Cr−DNA binding, the presence or the absence of dG in oligos did not have a significant effect on the extent of Asc−DNA cross-linking (Figure 7B).

Assessment of Ascorbate−DNA Cross-Links in Cells. Human cells are unable to synthesize ascorbic acid due to deficiency in L-gulono-γ-lactone oxidase which catalyzes the terminal step in Asc biosynthesis. A potential source of this vitamin for many human cells in culture is serum since several types of commonly used media do not contain Asc (F10, F12, L-15, MEM, DMEM, and RPMI 1640). Asc is readily oxidized at physiological pH and temperature, particularly in the presence of residual amounts of redox active metals iron and copper. DHA, the oxidized form of vitamin C, undergoes further rearrangements, producing physiologically inactive L-erythroascorbic acid (*36*). Therefore, it can be expected that commercial serum is not a very good source of Asc for cultured cells. In agreement with this prediction, we found no detectable Asc (<0.5 μM) in human A549 cells that were grown under standard conditions (90% F12 medium and 10% serum) using different batches of serum. These results were obtained using a sensitive and Asc-specific HPLC assay that quantified the amount of the fluorescent DHA−OPDA derivative. To create physiological concentrations of Asc in A549 cells, we initially used the reduced form of vitamin C that was added to the tissue culture medium for various periods of time (Figure 8). However, accumulation of Asc in A549 cells was low even after incubation for 24 h. In contrast, addition of DHA for only 90 min resulted in the significant accumulations of Asc in cells. The amount of transported DHA after 90 min was on average 10 times higher than that of Asc after incubation for 24 h. These large differences in the uptake of oxidized and reduced forms of vitamin C by A549 cells are similar to results reported for other types of cells (*37*). Once inside cells, DHA is rapidly (within minutes) reduced to Asc via glutathione-independent reactions (*37*). We observed that prolonged incubations or the use of high DHA concentrations frequently led to cytotoxicity detected 24 h after the incubations. In all subsequent experiments, we used nontoxic pretreatment with 2 mM DHA for 90 min, which resulted in an intracellular Asc concentration of 0.72 mM. It should be noted that A549 cells were not unusual in their intracel-

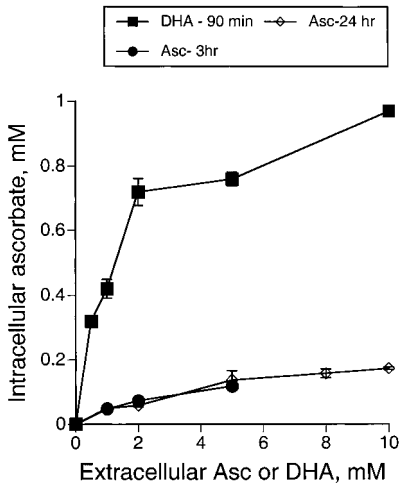


FIGURE 8: Uptake of ascorbate and dehydroascorbate by A549 cells. Cells were incubated with DHA for 90 min in Krebs buffer supplemented with 0.5 mM glucose. Incubation with Asc was performed in complete medium for 3 or 24 h. Intracellular concentrations of Asc were determined by HPLC detection of the fluorescent DHA−OPDA conjugate.

lular concentration of vitamin C or uptake of Asc and DHA in comparison to other human cells. For example, epithelial HeLa cells (DMEM/10% serum) and HCT116 cells (DMEM-F12/10% serum), as well as foreskin diploid fibroblasts (DMEM/10% serum), all had undetectable intracellular Asc. HeLa and HCT116 cells also exhibited rapid accumulation of DHA but not Asc (uptake by fibroblasts was not examined).

To determine whether Asc adducts can be formed in vivo, we purified DNA from A549 cells preloaded with Asc and then treated with Cr(VI). DNA was incubated with a phosphate/DPTA solution to dissociate DNA-bound Asc, and the amount of DHA−DDB derivative was quantified by HPLC with fluorescent detection. Although the DDB-based procedure recovered only 40% of Asc and it was more time-consuming than the HPLC assay with OPDA, the sensitivity of the former was much higher (detection limit of 20 fmol). Figure 9 shows that control cells (panel A) and cells preloaded with Asc (panel D) had identical background profiles in the absence of Cr(VI) exposure. The addition of Cr(VI) to DHA-preloaded A549 cells for 3 h led to a dose-dependent increase in the size of the Asc peak (Figure 9D−G). Exposure to Cr(VI) in the absence of DHA pretreatment did not result in the appearance of an HPLC peak corresponding to the Asc standard (Figure 9B). To test whether the cross-linking reaction could occur during the purification of DNA, lysates from Cr(VI)-exposed and DHA-free cells were spiked with 30 μM Asc (corresponds to an intracellular concentration of 1 mM) and DNA was purified via the standard procedure. These samples produced no detectable Asc signal relative to control cells (Figure 9C). We found that isolation of cellular DNA by the proteinase K/phenol procedure resulted in the recovery of 40.3% of the initial amount of Asc−DNA cross-links. This was determined by processing a known amount of Asc−DNA standards through the entire phenol/proteinase K procedure in each batch of the cellular samples. The trypan blue assay did not detect a significant loss of viability in any treatment group at the time of cell collection. The yield of Asc−DNA cross-links in Cr(VI)-exposed cells was linear over the entire range of concentrations (Figure 10). The linear dose response and cross-linking of as many as 1.3 Asc molecules/10000 bp of DNA after exposure to 10 μM Cr(VI) suggest that the formation of Asc−DNA cross-links is not likely to be a high-dose phenomenon. Three independent preparations of DNA obtained from cells following their exposure to 50 μM Cr(VI) were analyzed for both total Cr−DNA adducts and Asc−DNA cross-links. On the basis of these measurements, we calculated that Asc−Cr(III)−DNA cross-links made up 6.0 ± 1.0% of the total DNA adducts.

Mutagenicity of Ascorbate−Cr(III)−DNA Cross-Links. We employed a pSP189-based shuttle-vector approach to examine the mutagenicity of Asc−Cr(III)−DNA cross-links (*38*). Control and cross-link-containing pSP189 plasmids were replicated in human HF/SV fibroblasts, and the frequency of mutational events at the *supF* gene was determined by scoring arabinose-resistant colonies in an indicator *E. coli* strain (*39*). We found that cross-linking of 5.2 Asc molecules/1000 bp of pSP189 DNA led to a 15-fold increase in mutation frequency over control (Figure 11). Blocking of Asc−DNA cross-linking in reaction mixtures containing phosphate resulted in the background mutation frequency, indicating the absence of nonspecific mutagenic DNA damage in reactions with the Cr(III)−$(Asc)_2$ complex. This conclusion was further supported by findings that dissociation of approximately 90% of the Asc−DNA adducts by postincubation with 50 mM phosphate led to an 86.2% decrease in mutation frequency (Figure 11, reversal samples). The observed level of induction of mutations by Asc−DNA cross-links appeared to be much higher than that found for a similar number of binary Cr(III)−DNA adducts (*25*), suggesting that a bulky Asc ligand increases the mutagenicity of Cr−DNA adducts.

DISCUSSION

Reduction of Cr(VI) at neutral pH in the presence of physiological and subphysiological concentrations of Asc was found to produce Asc−DNA cross-links. Sequestration of Cr(III) by EDTA completely blocked the formation of Asc−DNA adducts, whereas incubation of preformed cross-links in the presence of inorganic phosphate dissociated Asc from DNA. These results along with the known ability of Cr(III) to form stable coordinate complexes with Asc (*17*) indicate that the Asc-containing adduct is a ternary Asc−Cr(III)−DNA complex. Reduction of Cr(VI) is also known to yield significant amounts of Asc$^{\bullet}$ radical derived primarily from the reduction of Cr(IV) to Cr(III) (*16*). We believe that a direct addition of Asc$^{\bullet}$ radical to DNA bases producing a cross-link is very unlikely for the following reasons. (i) Asc is very unreactive toward other molecules and decays through the reaction of disproportionation with another Asc$^{\bullet}$ radical (*40*). (ii) The reaction of addition would produce an adduct resistant to dissociation by Cr(III)-chelating phosphate, but the opposite was observed. In agreement with other recent studies on Cr(III)−DNA binding (*20*, *41*), the formation of both binary and ternary adducts showed no apparent preference for G- or A-containing oligonucleotides. Similar formation of Cr(III) adducts on single-stranded and double-stranded DNA suggests that the sites of adduct formation in a DNA duplex are not sterically hindered. The lack of base specificity, an inhibitory effect of Mg ions, and the known preference of Cr(III) for the negatively charged oxygen groups (*42*) all

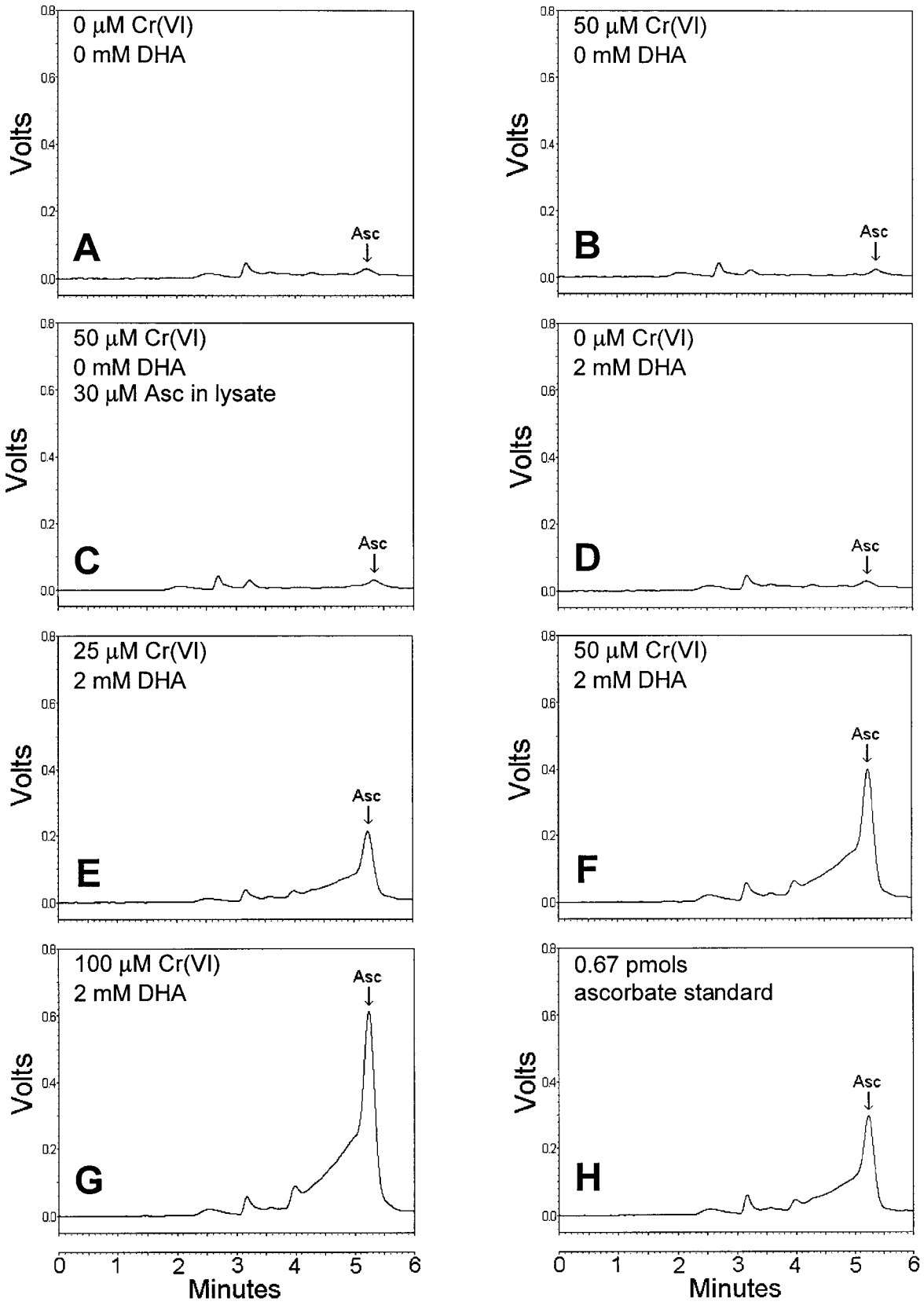


FIGURE 9: HPLC profiles of DNA samples from control and Cr(VI)-exposed A549 cells. Cells were incubated with 2 mM DHA in Krebs buffer with 0.5 mM glucose for 90 min, washed with PBS, and then exposed to 0−100 μM Cr(VI) in serum-free medium for 3 h. DNA was isolated by a proteinase K/phenol procedure. DNA-bound Asc was released by incubation in the phosphate/DPTA buffer, oxidized into DHA, and then derivatized with DDB. The fluorescent DNA−DDB derivative was detected using HPLC: (A) untreated A549 cells, (B) cells exposed to 50 μM Cr(VI), (C) lysates from cells treated with 50 μM Cr(VI) and supplemented with 30 μM Asc (corresponds to 1 mM Asc in intact cells) and then processed through the standard DNA isolation procedure, (D−G) cells pretreated with 2 mM DHA and then exposed to 0, 25, 50, and 100 μM Cr(VI), respectively, and (H) 0.67 pmol of authentic Asc.

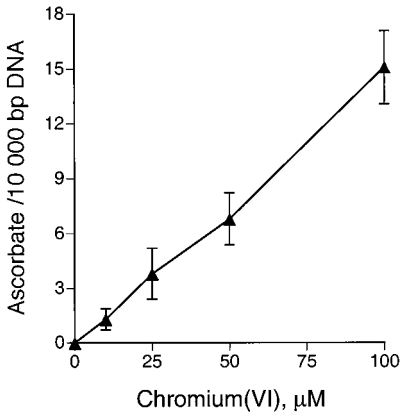


FIGURE 10: Dose-dependent formation of ascorbate−DNA cross-links in A549 cells. Cells were pretreated with 2 mM DHA for 90 min in a Krebs/0.5 mM glucose solution and then exposed to Cr(VI) for 3 h in serum-free medium. The amount of DNA-bound ascorbate was quantified as described in the legend of Figure 9. Shown are means ± SD from four independent preparations of DNA.

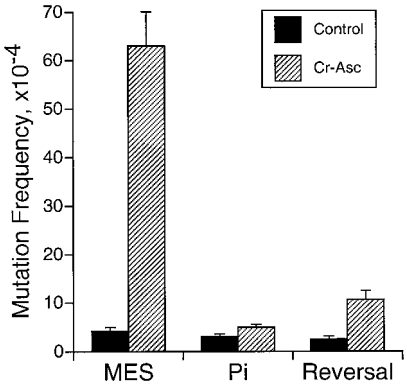
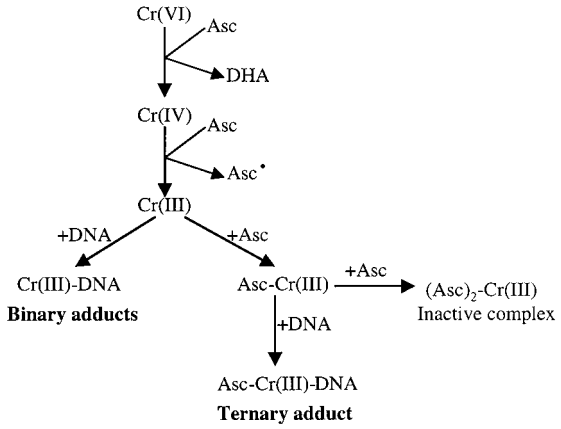


FIGURE 11: Mutagenicity of ascorbate−Cr(III)−DNA cross-links in human fibroblasts. Cross-links were formed by reacting 0.5 mM Cr(III)−$(Asc)_2$ complex with pSP189 DNA in 25 MES buffer at pH 6.0 (5.2 Asc molecules/1000 bp of DNA). The potential formation of nonspecific DNA damage by the Cr(III)−$(Asc)_2$ complex was examined in reaction mixtures containing 25 mM phosphate (pH 6.0) (no cross-links). Dissociation of Asc−DNA cross-links was performed by incubation in 50 mM phosphate (pH 7.0) for 24 h at 4 °C (10% remaining cross-links). Control and modified pSP189 DNA were propagated in HF/SV human fibroblasts, and plasmids were isolated and then scored for the presence of mutations at the *supF* gene. For MES data, cross-link-producing reactions in MES buffer ($n = 6$); for Pi data, cross-link-blocking incubations in phosphate buffer ($n = 2$). For Reversal, 90% of the Asc−DNA cross-links formed in MES buffer disrupted by a subsequent incubation in phosphate ($n = 2$). Shown are means ± SD.

indicate that the DNA phosphates were the principal sites of adduct formation. The attachment of Cr(III) to the 5′-phosphate group can potentially lead to the formation of a second coordinate or hydrogen bond to N7-G which is considered to be important for the induction of mutagenic effects (*26*). The pattern of arrest sites produced during in vitro replication of Cr-modified templates is consistent with the presence of additional Cr(III)−dG interactions in a subset of DNA adducts (*21*). It remains to be determined which type of Cr−DNA adducts involves binding to dG groups.

Other investigators (*15*) have also looked at the possibility of Asc−DNA cross-linking using [^{14}C]Asc and reported the lack of DNA-associated radioactivity. It appears that the previous efforts were focused on the reactions with the low



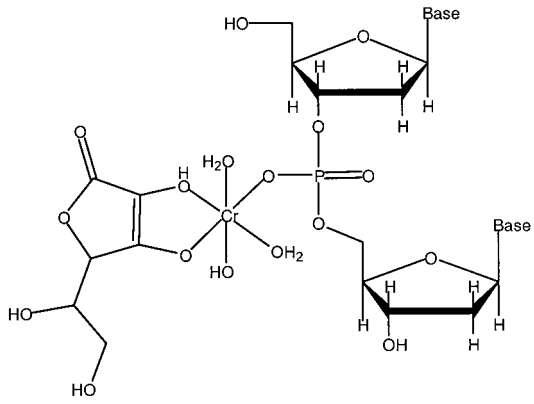


FIGURE 12: Proposed mechanism of ascorbate−Cr(III)−DNA cross-linking. Cr(III) coordination to Asc O-3 is based on spectroscopic and other evidence (*17*, *44*−*46*), whereas the formation of the chelate ring at O-2 is suggested by the stability of Cr(III)−Asc complexes (*45*) and geometric considerations (*47*).

ratios of Asc to Cr(VI), which is expected to result in almost complete oxidation of Asc to DHA. However, DHA is not capable of forming stable coordinate complexes with metals (*17*, *35*) which could at least partially explain the absence of DNA-bound radioactivity. We also found that commercial preparations of [^{14}C]Asc contain large amounts of oxidized products, which leads to a low sensitivity and variable measurements.

Human cells in vivo contain a millimolar concentration of Asc (*43*), which means that this reducer will be present in a large excess over gradually entering Cr(VI). Reduction of Cr(VI) under these conditions proceeds through the initial transfer of two electrons, producing Cr(IV) as the first intermediate form (*14*−*16*). The absence of Cr(V) in these reactions, the lack of Cr(V)−DNA ESR signals even in the reactions with 0.5 mM EHBA−Cr(V) complex (*41*), and extensive formation Cr−DNA adducts in our experiments with biologically relevant ratios of the reactants all indicate that Cr(V) is not needed for the formation of either binary or ternary DNA complexes. We did observe a somewhat higher level of total Cr−DNA binding in the presence of 0.2 mM Asc in comparison with 2 mM-based reactions, but this was a relatively small effect which most likely reflects the ability of Asc to compete with DNA for the reactive Cr(III) complexes. A very low reactivity of Cr(III)−$(Asc)_2$ complexes was directly confirmed in this work. The ability of Cr(III) chelating agents, EDTA and inorganic phosphate,

to block the formation of DNA adducts suggests the importance of this final oxidative form in the production of DNA damage. If Cr(V) or Cr(IV) were first coordinated to DNA and then reduced to Cr(III) in its DNA-bound form, neither phosphate nor EDTA would be able to dissociate these adducts with an incubation time of 5−30 min. We determined that it takes at least 24 h to release 80% of the binary and 90% of the ternary adducts. Moreover, the majority of Cr(III)−DNA adducts are stable in the presence of EDTA (ref *29* and this work). The proposed mechanism for the formation of binary and ternary adducts is presented schematically in Figure 12. Competition between the negatively charged DNA polymer and Asc for reactive Cr(III) centers is expected to be the main determining factor in the relative yield of each type of adduct. The inability of DNA-bound Cr(III) to react with Asc is probably caused by the difficulties in establishing a close metal−ligand contact due to electrostatic repulsion between negatively charged Asc and DNA polyanion.

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BI011942Z