Mechanisms of metal ion mutagenesis
by Barbara Annette Vieux

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University

Abstract:
The recently derived ochre hisG428 strains TA102 and 103 from Salmonella typhi-murium are particularly useful in the analysis of the mutagenic activity of substitutionally inert transition metal complexes as well as the CrVI compound, potassium dichromate. Because of the fortuitous location of the hisG428 mutation in the structural G gene, rever-tants will either retain or lose the feedback inhibition site. Therefore, different categories of revertants have a differential sensitivity to the histidine analog, thiazolealanine. The histidine analog allows the identification of the induced mutations into three categories: (a) small deletions, (b) base pair mutations, and (c) extragenic suppressors with TA102. Induced mutations in TA103 only result in categories b and c.

Because of the variability inherent in a sensitivity disc assay, we have developed a microtiter suspension assay system that can quantitatively relate analog concentration to growth inhibition of the particular category of mutant.

In addition to establishing the mutagenic property of both CrIII and CrVI compounds, we have categorized the different apparent classes of mutations. Dichromate (CrVI) induces mainly point revertants while CrlII, cis[Cr(bipy)2Cl2] induced predominantly extragenic suppressors of TA103. PtlI, cis[Pt(NH3)2Cl2], induced about equal numbers of extragenic suppressors and point revertants while PtIV, cis[Pt(NH3)2Cl4] induced point revertants in TA103. Ptll and PtIV induced about an even distribution of all three types in TA102.

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Barbara Annette Vieux

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ABSTRACT

The recently derived ochre hisG428 strains TA102 and 103 from *Salmonella typhimurium* are particularly useful in the analysis of the mutagenic activity of substitutionally inert transition metal complexes as well as the CrVI compound, potassium dichromate. Because of the fortuitous location of the hisG428 mutation in the structural G gene, revertants will either retain or lose the feedback inhibition site. Therefore, different categories of revertants have a differential sensitivity to the histidine analog, thiazolealanine. The histidine analog allows the identification of the induced mutations into three categories: (a) small deletions, (b) base pair mutations, and (c) extragenic suppressors with TA102. Induced mutations in TA103 only result in categories b and c.

Because of the variability inherent in a sensitivity disc assay, we have developed a microtiter suspension assay system that can quantitatively relate analog concentration to growth inhibition of the particular category of mutant.

In addition to establishing the mutagenic property of both CrIII and CrVI compounds, we have categorized the different apparent classes of mutations. Dichromate (CrVI) induces mainly point revertants while CrIII, cis$[Cr(bipy)_2 Cl_2]$ induced predominantly extragenic suppressors of TA103. PtII, cis$[Pt(NH_3)_2 Cl_2]$, induced about equal numbers of extragenic suppressors and point revertants while PtIV, cis$[Pt(NH_3)_2 Cl_4]$ induced point revertants in TA103. PtII and PtIV induced about an even distribution of all three types in TA102.

INTRODUCTION

The health implications of mutagenic agents in our environment have been recognized, albeit gradually, since the middle fifties. Early experiments in examining the biological effects of ionizing radiation led the general population to believe that the damaging capabilities of ionizing radiation could lead to permanent changes or mutations in the DNA. Thus basic research was conducted by many laboratories into the detailed mechanisms of radiation damage of DNA. The public was also made aware of the need to control the unnecessary exposure to X-rays, ionizing radiation from radioactive decay and UV irradiation.

During the middle sixties, health researchers gradually became aware that there was a connection between lifestyle, intrinsic factors, extrinsic factors and the probability of incurring some type of cancer.

The concept that cancer could be in large part an avoidable disease was recognized by the World Health Organization (WHO). In 1964 this organization came forth with the following statement: "The potential scope of cancer prevention is limited by the proportion of human cancers in which extrinsic factors are responsible. These [factors] include all environmental carcinogens (whether identified or not) as well as 'modifying factors' that favor neoplasia of apparently intrinsic origin (e.g., hormonal imbalances, dietary deficiencies and metabolic defects). The categories of cancer that are thus influenced, directly or indirectly, by extrinsic factors include many tumours of the skin and mouth, the respiratory, gastrointestinal and urinary tracts, hormone dependent organs (such as the breast, thyroid and uterus), haematopoietic and lymphopoietic systems, which, collectively,

account for more than three-quarters of human cancers. It would seem, therefore, that the majority of human cancer is potentially preventible" [1].

In spite of the fact that certain chemicals were known to damage DNA and thus were mutagenic and had the same potential of being harmful to human health, it was not until the early seventies that the mutagenicity of certain chemicals became an important toxicological property to be considered from a regulatory point of view.

During this same time period there was speculation that a mutation could initiate an event in a cell that after an undetermined period of time could lead to that cell becoming a tumor cell. This was called the somatic cell mutation theory of cancer. To this end Bruce Ames has been developing an assay for the detection of mutagenic chemicals using the Salmonella bacterial system. In December of 1975 Ames and colleagues published a paper demonstrating the utility of the Ames system in detecting the mutagenicity of chemicals. The three hundred compounds tested included both known carcinogens and known non-carcinogens. With the compounds tested, the Ames test was approximately 90% reliable in predicting whether or not a particular compound would be carcinogenic [2]. Since the only other methodologies available for identifying carcinogens are through very expensive and time consuming animal tests, the Ames test could be a revolutionary development in cancer research. The Ames test as a predictive tool has been recently evaluated and was found to be the most useful of all the bacterial tests and to be about 80% accurate in correlating carcinogenicity in rodents with mutagenicity in the Ames test. This is a remarkably high predictability since the standard Ames strains involve the reversion of a GC base pair in two different loci by two different mechanisms. A major apparent deficiency of the Ames system was the inability to demonstrate reversion by putative carcinogenic inorganic compounds [29].

Statement of Problem

The purpose of this thesis is to:

A. Compare the efficacy of certain inorganic compounds and substitutionally inert complexes in reverting the Standard Ames strains with the new Levin-Ames strains. There has been a considerable amount of controversy as to the mutagenic properties of CrIII and CrIV. With the new Levin-Ames strains with an AT reversion site, I planned to retest CrIII for mutagenic activity.

B. Compare the mechanism of reversion of certain inorganic compounds such as potassium dichromate and substitutionally inert complexes such as cis$[Cr(Bipy)_2 Cl_2]$ using the new Levin-Ames strains. This new system which compares the mechanisms of reversion may facilitate screening for potential anti-tumor drugs with similar properties to cis dichlorodiamine PtII.

HISTORICAL DEVELOPMENT OF STANDARD AMES STRAINS

By isolating mutants which had an inactivated gene for histidine biosynthesis, strains were selected for histidine autrophy. Mutants were selected from two categories, base pair substitution and frameshift mutations. Of the five standard tester strains, two detect base pair substitution and three detect frame shift mutations.

With base pair substitution, one nucleotide which is normally present is replaced by another. The original defect in this gene is caused by the substitution of cytosine for a thymine. Instead of coding for leucine, it now codes for proline (Figure 1). Proline causes a large kink in the protein chain, rendering it nonfunctional. However, almost any base change which results in the coding for any other amino acid besides proline to be coded for will allow the protein to function. This contributes to the sensitivity of these strains of *S. typhimurim* to a wide variety of base pair substitution mutagens. Then the original wild-type code —C—T—C— (leu), or other mutations such as —A—C—C— (thr), —T—C—C— (ser), —C—A—C— (his), initiates histidine synthesis [3].

In a frameshift mutation, if one or two nucleotides are either added or deleted (called a lesion), the nucleotides downstream from the mutation are mismatched. There is a literal shifting of the DNA base template. Mechanistically, the process of inserting or deleting one or two base pairs results in a nucleotide sequence which alters the entire primary sequence of the protein downstream from the lesion (Figure 2) [1,3].

The original mutation in the frameshifter strain contained a deletion of a single cytosine which resulted in a shift of the normal reading frame. All codes downstream change as the nucleotide base is shifted by one position. This occurs frequently in a hotspot region, C—G—C—G—C—G—C—G—, where slippage can occur easily. Reversion to a his+ mutation

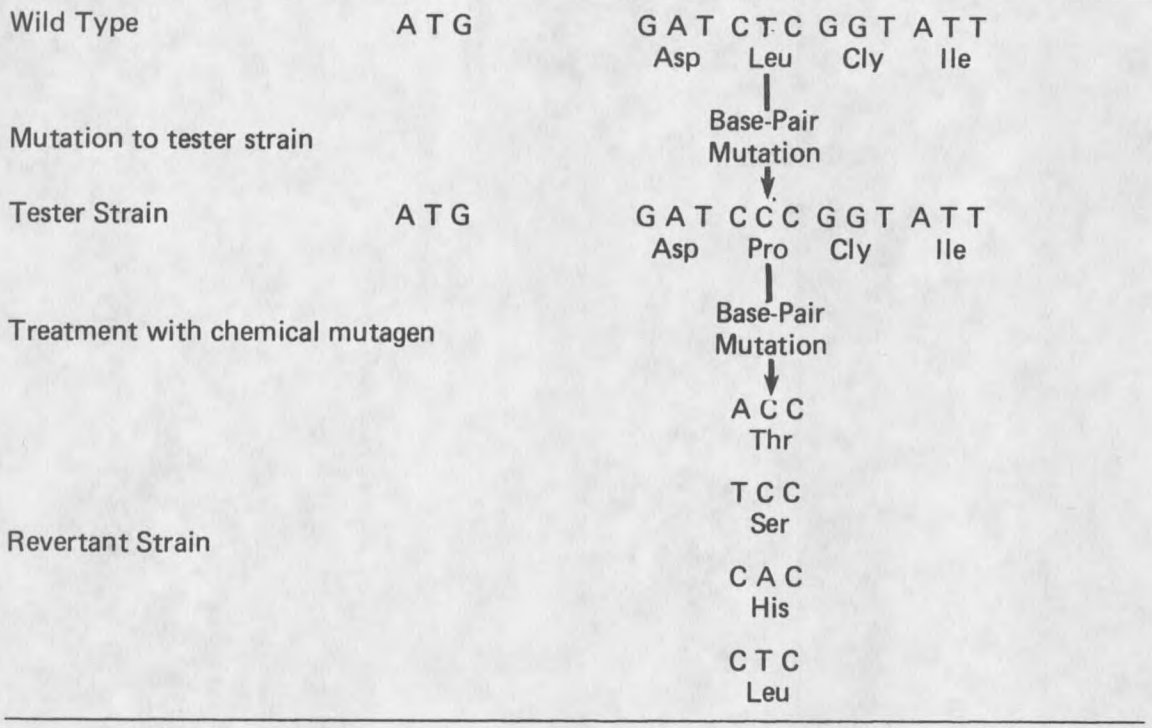


Figure 1. Base-pair substitution mutations in the *S. typhimurium* histidine gene.

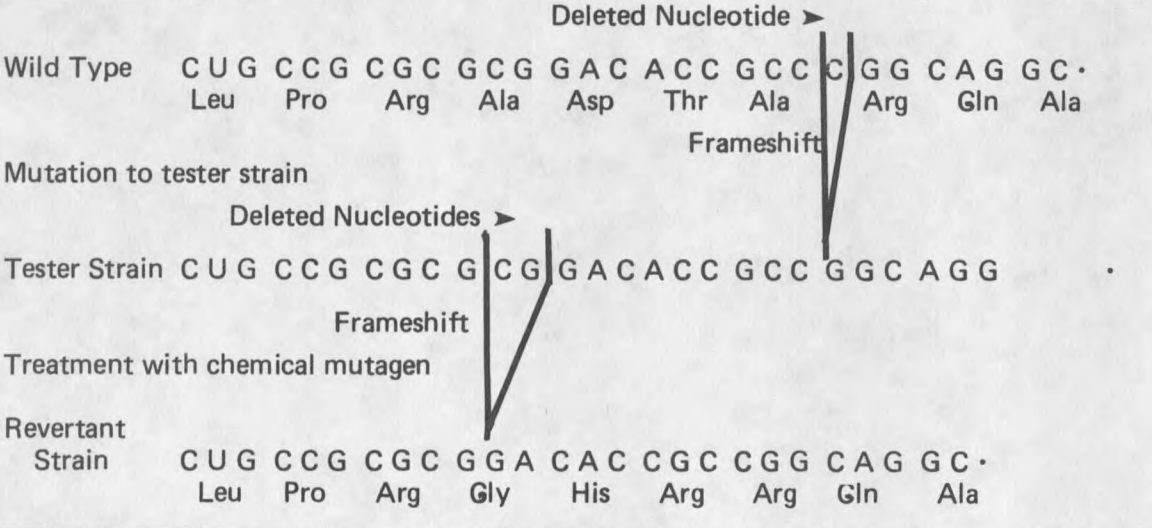


Figure 2. Frameshift mutations in the *S. typhimurium* histidine gene.

occurs with the deletion of a C—G base pair within this region which puts the reading frame back into proper reading order. The result is a gene product with only a very short region of incorrect codes. Usually, the protein generated from this gene can still function and histidine is produced.

It has been demonstrated that many chemicals are not direct acting as outlined above, but can be metabolized into carcinogenic forms through activity of microsomal liver enzymes [4,5]. If a compound is mutagenic, the active chemical randomly reacts with bacterial DNA. When this occurs at or near the original mutation site of the tester strain, a second or reverse mutation may results. The histidine gene is converted to a functional structure. This allows resumption of histidine biosynthesis and visible growth of revertant colonies on histidine limited media. When microsomal liver extracts are added to the Ames test, the mammalian capacity to metabolize potential carcinogens into their active forms allows for the detection of indirect acting carcinogens (such as the aromatic amines and polycyclic hydrocarbons).

Operon Regulation of Histidine Synthesis

Biosynthesis of histidine is regulated at both the transcriptional level and by feedback inhibition. The histidine operon in Salmonella has been shown to function without a regulatory protein. Instead, the expression of this cluster of nine genes, required for production of histidine, is controlled by sensing the level of histidyl-tRNA. The control region is found to contain a sequence which codes for seven adjacent histidine codons. These codons can provide a sensitive means for determining the concentration of histidyl-tRNA.

HisO mutations in the control region showed that there is a barrier to transcription at the attenuation site [6]. This site was altered by the hisO1242 deletion. Transcription

can continue through the attenuator and into the structural genes. However, this occurs only when translation occurs simultaneously.

DNA sequencing shows that there is dyad symmetry which permits the formation of an attenuator site with a 14 base-pair stem and loop in mRNA. This stem includes a rich G—C region followed by 9 uridyl residues. These residues are associated with message termination signals in other systems. This attenuation model is similar to one described for the trp operon [7].

It is assumed that one particular attenuator stem can prevent formation of a later alternative stem, even if the second stem is energetically favored. This occurs if the time involved to shift from the first stem to the second is so great that RNA polymerase would pass the critical point for termination before the attenuator stem could form.

Message termination and regulation is mediated through translation of the leader peptide. The ribosome appears to be the major positive factor in regulation even though some proteins may stabilize loop structures of many operons. The model predicts that the termination of protein synthesis preceding the run of his codons would cause the formation of the attenuator stem and lead to transcription termination.

The his operon is expressed at basal level under maximally repressed conditions. When the attenuator stem appears capable of total repression, expression may be due to the position of the ribosome. Occasionally the first ribosome is late in initiating leader peptide synthesis or is slowed by a fluctuation in the concentration of any of the charged tRNAs. Under these conditions the attenuator would not form. Ribosomes probably do not "arrest" but rather are slowed so that they are separated from polymerases. This will influence various stem formations in the mRNA secondary structures. Therefore, in the his+ revertants, the production of histidine is regulated by the mechanism of attenutation.

Standard Strains

The standard Ames' strains are sensitive to a wide range of mutagens due to the different types of mutations in the histidine gene, the presence of mammalian liver homogenate, and various other factors such as mutations in various DNA repair mechanisms (Table 1). Three mechanisms by which the cell may repair damaged DNA are: (1) direct enzymatic reversal of altered DNA to its original form; (2) specific removal of the damaged DNA by excision enzymes followed by resynthesis of the excised portion; and (3) the deletion of damaged DNA through a series of enzymatically-mediated recombination events to produce at least one "good" copy of the DNA [5]. Mechanisms 1 and 2 are categorized as error free repair and 3 is referred to as error prone repair due to recombination and SOS processing.

Table 1. Genotypes of the Standard Strains used for Mutagen Testing.

Histidine Mutation			Additional Mutations		
hisG46	hisC3076	hisD3052	LPS	Repair	R factor
TA1535	TA1537	TA1538	*rfa*	Δ*uvrB*	–
TA100		TA98	*rfa*	Δ*uvrB*	+R
(TA1975)	(TA1977)	TA1978	*rfa*	+	–
hisG46	hisC3076	hisD3052	+	+	–
TA92		TA2420	+	+	+R
TA1950	TA1952	TA1534	+	Δ*uvrB*	–
TA2410			+	Δ*uvrB*	+R
TA1530	TA1532	TA1964	Δ*gal*	Δ*uvrB*	–
TZ2631		TA2641	Δ*gal*	Δ*uvrB*	+R
	TA2637		*rfa*	Δ*uvrB*	+R

The *uvrB* gene codes for one of the groups of enzymes of the excision repair system. If this gene is deleted, the mutability of the bacteria increases and it becomes more sensitive to weak mutagen activity.

The *rfa* mutation results in a defect in the thick lypopolysaccharide outer coat of the bacteria. This mutation allows for large organic molecules to diffuse more readily into the bacteria.

Another refinement of the system concerns the error prone DNA repair system. This is a 'well-meaning' system for correcting some types of DNA damage with frequent coding errors. If one incorporates the activity of this system, one can increase the detection of very weak mutagens. pKM101 is an introduced, multicopy plasmid which codes for this repair system. This plasmid has broad effects. For example, the mutagen aflatoxin-B_2 is extremely carcinogenic in animals but barely mutagenic in the standard protocol of the Ames test in bacterial systems without the pKM101 plasmid. This plasmid gives enhanced detection of base-pair substitution mutagens and frame shift mutagens.

New Strains

The new Ames tester strains have A–T base pairs at the site of mutation in contrast to the standard strains that detect mutagens damaging G–C base pairs. These are labeled TA102, 103, 2638, and 104 (Table 2). The mutants are of the ochre type at the A–T base pair and this mutation can be suppressed by strains that carry ochre suppressors. The mutation sequence at positions 839-853 is A–G–A–G–C–A–A–G–T–A–A–G–A–G–C as opposed to the wild type with the sequence A–G–A–G–C–A–A–G–C–A–A–G–A–G–C [8]. The TAA triplet is transcribed as a stop codon. This confirms the classification of this mutant as an ochre type with the presence of only A–T base pairs in the mutated triplet.

This particular strain, hisG428, was selected for its extreme sensitivity to oxidizing mutagens. Using the new strains containing the hisG428 mutation, David Levin has developed an assay which can be used to determine the mechanism of induced and spontaneous reversions.

Table 2. Genotype of the New Strains used for Mutagen Testing.

Strain	Genotype
LT2	Wild type
SA2197	purC7/F'42 fin-301 lactose-utilizing
hisG428	hisG428
TA2892	hisΔ(G)8476/F'42 fin-301 lactose-utilizing
TA2665	hisΔ(G)8476
TA2661	hisΔ(G)8476/pAQ1
TA2662	hisΔ(G)8476/pAQ1/pKM101
TA2898	hisG428 *recA1 srl-2*::Tn10
TA2899	hisG428 *recA1 srl-2*::Tn10/pKM101
TA1890	zec-2::Tn10 hisG428 polA2 ara-9
TA1891	TA1890/pKM101
TA2659	hisG428 Δ*uvrB gal bio chl-1057 rfa-1028*
TA104	TA2659/pKM101
TA103	hisG428/pKM101
TA2657	hisΔ(G)8476 *galE503 rfa-1027*/pAQ1
TA102	TA2657/pKM101
TA2638	hisG428 *galE531 rfa-1026*/pKM101

Levin et al. described a simplified, quantitative method for detecting small base pair deletions of 3 or 6 base pairs [9]. Using DNA sequence analysis of the hisG gene in spontaneous revertants of TA103 of the ochre (TAA) mutation, Levin found that the mutant can revert back to the wild type by base pair substitution and produce glutamine, or by reverting the ochre triplet to code for a lysine or a leucine. Small base pair deletions maintain the reading frame while removing the ochre triplet (Figure 3).

The hisG428 site is apparently a critical determinant for feedback inhibition of the encoded enzyme. Deletion of one or two amino acids from the protein at this position results in an enzyme that is not subject to feedback inhibition by the histidine analog, thiazolealanine. A third major class of revertants were found to be hypersensitive to thiazolealanine inhibition. These revertants contain extragenic ochre suppressor mutations residing in the anticodons of four tRNA species (Levin, personal communication). The hisG428 site is flanked by 5 base pair repeats of 5'—A—G—A—G—C—3' which may be important in generation of small deletions through base mispairing. This may also allow for

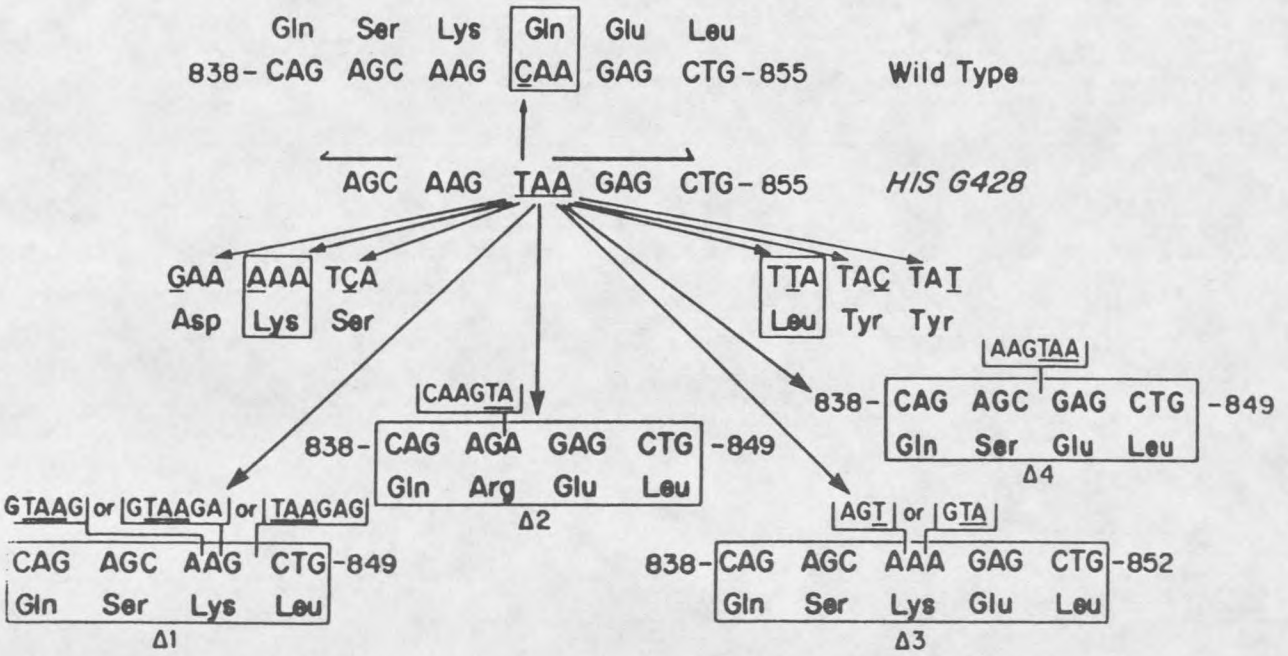


Figure 3. DNA sequence analysis of spontaneous revertants of TA103[hisG428/pKM101]. Base pair substitution mutations were to glutamine (CAA, strain TA2668), leucine (TTA, strain TA-2669), and lysine (AAA, strain TA2670). Deletion revertants 1 through 4 were designated TA2671, TA2672, TA2673, and TA2674, respectively [9].

persistence of a single strand region of DNA at this site which may be more sensitive to damage by mutagenesis.

The mechanisms of deletion formation were studied by Albertini and Miller [10]. Large deletions of 700-1000 base pairs long were identified by using lacI-Z fusion strains of *E. coli*. These deletions occurred between short homologies of as few as 5-8 base pairs. It appears that the surrounding sequence and even the nature of the repeated sequence itself affects the deletion frequency (Figure 4). Streisinger proposed that slipped mispairing during DNA synthesis may be involved in deletion formation and it has also been noted that spontaneous repeats often are associated with spontaneous deletions [11]. The *recA* protein may be a principal enzyme involved in recombinational repair events that result in deletions with the slipped mispairing being catalyzed by the *recA* protein [10].

Levin looked at the frequency of spontaneous hisG428 deletion revertants in different genetic backgrounds. The error-prone repair system is encoded in the pKM101 plasmid [9]. This system greatly increases the frequency of spontaneous revertants due to point mutations, but not in revertants due to deletions.

When the *recA* gene was added to a strain (TA2899), the increase in spontaneous reversion frequency due to pKM101 is abolished. This gene is also required for error prone repair processes and is implicated in SOS dependent mutagenesis. Miller found that large deletions were detected 25-fold more frequently in *Escherichia coli* with a *recA+* background [10].

The PolA mutation (lacking in polymerizing activity of DNA polymerase in TA1890) increased the frequency of spontaneous revertants by two fold. When pKM101 was added to a strain (TA2898), the frequency of spontaneous reversion was increased by a factor of 20.

The *uvrB* mutation (excision repair) in TA2659 and in *recA* gene in TA2898 both suppressed the generation of spontaneous revertants even with the pKM101. Mutants in

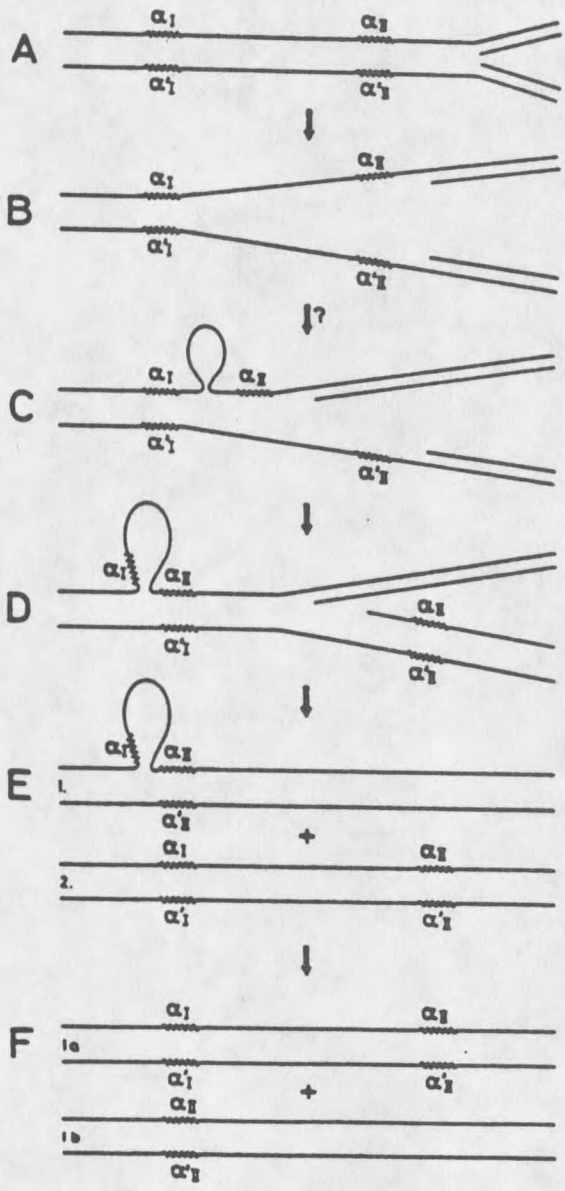


Figure 4. A schematic model for deletion formation by slipped pairing. Two homologous sequences, α_I and α_{II}, are indicated. During DNA replication, slipped mispairing, which might be prompted by inverted repeats interior to the two direct repeats (C), may occur, leading to deletion formation either upon either replication through the structures indicated here or by excision-repair of the looped region shown in (E1) [12].

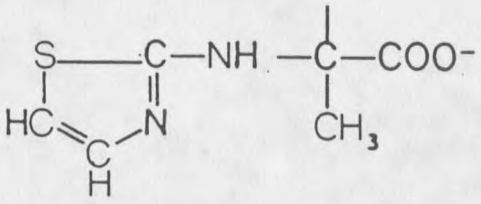
these related repair systems may be deficient in a common pathway involved in the generation of deletion mutations as *uvrB* is under control of *lexA* and *recA*.

Multiple copies of hisG428 on pAQ1 increased the frequency of spontaneous deletions by about 40 fold. This is in direct correlation with the number of copies of the gene of the plasmid. The strain TA102 contains the pAQ1 plasmid and are therefore more sensitive to the detection of deletions than in TA103 where the hisG428 gene resides solely on the chromosome.

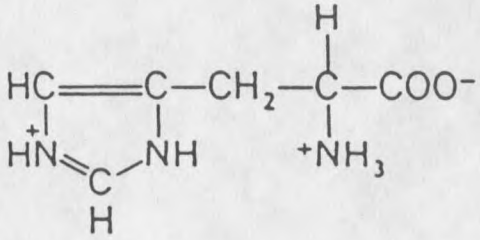
Deletion revertants can be characterized by the degree of resistance to the inhibitory histidine analog thiazolealanine (Figure 5). These compounds affect the feedback inhibition of the hisG gene product which catalyzes the first step in the biosynthesis of histidine. When three or six base pairs are deleted at the ochre mutation, the resulting enzyme remains viable and is not subject to feedback inhibition. These his+ revertants which are formed as a result of base pair substitution are sensitive in varying degrees to the analogs. Mutations due to extragenic suppressors are characterized by a hypersensitive response. A mutation of the t-RNA which resides on the anti-codon produces an extragenic suppressor. When exposed to one of the analogs, the cell starves for histidine and growth is inhibited.

Thiazolealanine is an inhibitory histidine analog which blocks the hisG gene product, phosphoribosyl-ATP-synthetase, at the feedback inhibition site. This enzyme (phosphoribosyl-ATP-synthetase) catalyzes the first step in the biosynthesis of histidine at the histidine feedback site regulating the biosynthesis of histidine.

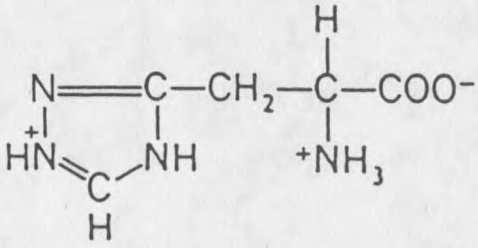
Triazolealanine affects extragenic suppressors to a greater degree than the histidine analog, thiazolealanine. This type of mutation on the t-RNA is hypersensitive to this compound. Triazolealanine, unlike thiazolealanine is incorporated into the his t-RNA and produces fake t-RNA which causes the cell to starve for histidine. In treating the *S. typhimurium* with the histidine analog triazolealanine, one cannot differentiate between deletions and point reversions. Triazolealanine may be used to confirm differentiation between



Thiazolealanine



L-Histidine



Triazolealanine

Figure 5. L-Histidine and analogs.

point reversions and extragenic suppressors when used with TA103. With this tester strain only 3% of the population of either spontaneous or induced revertants are attributed to deletions [8]. One may then assume that the hypersensitive colonies are due to extragenic suppressors and the remaining mutations to point reversions. The difference in deletion mutations between TA102 and 2638 indicate that disparate pathways exist for the generation of spontaneous and mutagen-induced revertants. Differences may exist in replicative DNA repair between the chromosome and pAQ1 plasmid. Alternatively the relative degree of supercoiling may be responsible for the differential sensitivity to mutagenesis.

The Levin assay shows three major classes of mutation. First, a three to six base pair deletion mutation that is resistant to the analog thiazolealanine, whereas the second major class of revertants is a point mutation sensitive to this analog. A third major class of revertants is hypersensitive to thiazolealanine inhibition. These revertants contain a mutation in the anticodon of the t-RNA.

Miller used purely genetic techniques to analyze amber and ochre mutations within the lacI gene in order to determine the sequence changes that occurred [12]. This method of determining the mechanism which causes a particular mutagenic event is much more time consuming than the Levin method.

Mutagen Induced Deletion Revertants

Mutagens and gyrase inhibitors induce deletions in TA102 and yet not in TA2638. The mutagenic agents which act by deleting nucleotides in TA102 revertants were not appreciably mutagenic on TA2657, which is a derivative of TA102 without pKM101. Therefore, it follows that pKM101 is required for the generation of mutagen induced deletion revertants. Bleomycin, an antitumor antibiotic, causes single strand breakage in DNA [9]. This may be due to the ability of bleomycin to generate hydroxyl radicals with a preferential release of free thymine adjacent to guanine in DNA [8]. X-rays cause single

and double strand breakage due to hydroxyl radicals. These may be premutagenic lesions responsible for mutagen-induced deletion revertants of TA102. Ultraviolet light forms thymine dimers. This activity can be detected on the hisG428 gene which has two adjacent thymines at the mutated site available for dimer formation. Nalidixic acid, Oxolinic acid and AM 715 show mutagenic activity corresponding to their ability to inhibit gyrase. These gyrase inhibitors produce primarily deletion revertants in TA102.

Targeted Mutagenesis

Theoretically, targeted mutagenesis would occur when the induced SOS processing system, which includes the *recA* protein, introduced mutations at specific sites of lesions in DNA. An untargeted event would introduce mutations at random sites regardless of whether or not there were lesions at those sites.

Although untargeted mutagenesis can occur on DNA that has not been damaged by an exogenous agent, it is now apparent that most of the mutations that arise after the treatment of *E. coli* with UV or chemical mutagens are the result of a targeted cellular process [13].

A mutagenic process could be locally targeted by the presence of a lesion with the introduction of a mutation at that site or in the immediate vicinity. Another mechanism could be due to a noncoding or pseudoinformational lesion due to apurinic sites or cyclobutane pyrimidime dimers [14].

Walker's studies with several SOS-dependent mutagens were all found to have their own characteristic spectrum of mutation, implicating targeted mutagenesis. If these were non-targeted mutations then the same spectrum of mutations would be generated by all of the mutagens.

Metal Mutagenesis

Platinum Compounds

The binding of cis DDP, dichlorodiamine PtII, has been found to be sensitive to the local DNA structure [15]. Studies with ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide) has shown that upon intercalation the DNA helix is known to unwind [16,17]. This alteration of the local structure of DNA could account for the enhanced effect of cis DDP on exonuclease III digestion in the d(G6-CG2) site when ethidium bromide is present in the Pt reaction mixture. It is thought that ethidium bromide could bind selectively to a certain region of the DNA duplex and enhance or initiate cis DDP binding in the vicinity [18].

Another theory is that bound ethidium bromide could change the mode of cis DDP binding to DNA by switching the Pt from a bidentate to monodentate coordination [19]. The binding region of cis DDP, d(GCGCG), crystallizes in a left-handed double helical form, Z-DNA. The rate of DNase I cutting to a given phophodiester bond of the dodecanucleotide depends on the angle of adjacent bases. This angle changes as the helix reforms as Z-DNA.

Cis DDP has been shown to unwind the double helix with formation of single strand of DNA [20]. Early studies suggest that cis DDP forms the most stable linkages with guanine at the N-7 position (Figure 6) [21,22]. It is stabilized by intramolecular hydrogen bonding between a coordinated water (or amine) ligand molecule and the exocyclic ring oxygen. This leads to weakened hydrogen bonds in the G—C pair that could lead to the denaturation of the double helix. A critical lesion leading to antitumor activity is a specific cross-link formed between two DNA binding sites by the Pt atom of cis DDP. With a low frequency of crosslinking DNA, the trans structure of DDP does not form this linkage.

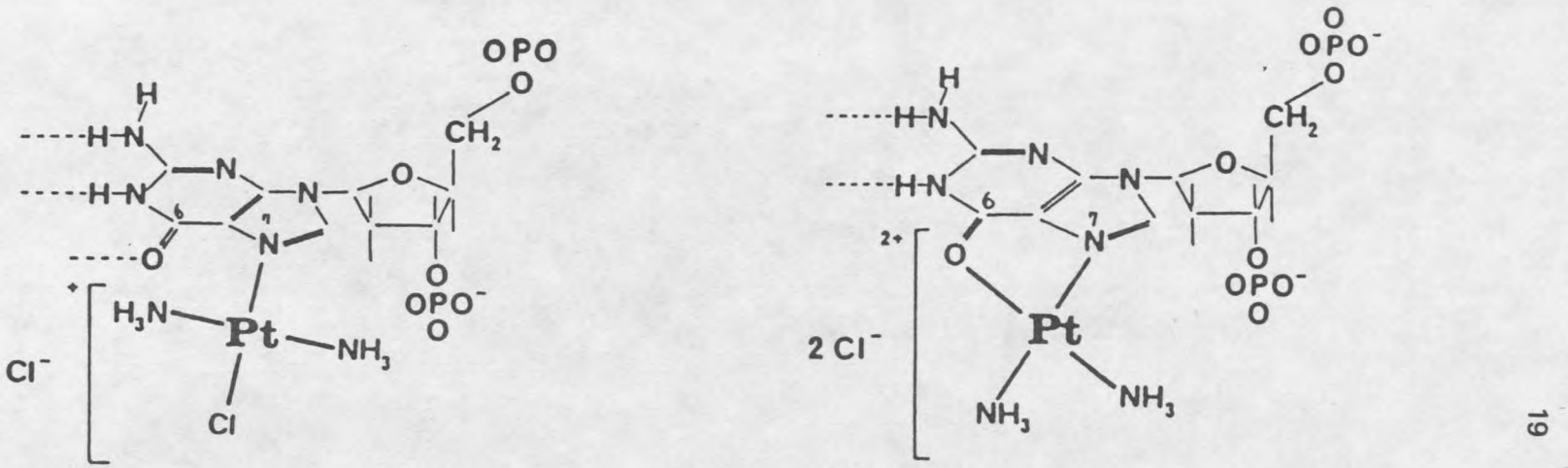


Figure 6. Hypothetical reaction products of cis and trans isomers of aquated diammineplatinum complexes with guanine. The cis isomer may form a closed-ring chelate complex between the N7-06 nucleophilic sites, while the trans isomer acts via a monodentate bond to N-7.

Andersen has shown that PtII generated frameshift mutations in TA98 and TA100, both strains have the R-factor plasmid [16]. This plasmid with the *recA* gene appears to be essential in detecting the mutagenic activity of platinum.

Das Sarma used a biochemical prophage induction assay to analyze the structural features of platinum compounds assuming that the relative induced capacity of the compound reflects its relative interaction with DNA [23].

Chromium Compounds

Using TA102, as it is particularly suited to the detection of oxidative mutagens, DeFlora has shown that the CrIII compounds, chromic acetate, chromic nitrate and chromic potassium sulfate have no mutagenic activity [24]. However TA102 did show mutagenic activity with Cr compounds in their oxidized state, i.e., CrVI.

Langerwerf et al. found CrIII with amino acid complexes not to be mutagenic using the standard Ames strains [5]. Using the new Levin-Ames strains with AT reversion site, Garland shows that CrIII is indeed mutagenic (see Figure 7).

Again, Pertilli and DeFlora found that CrVI is mutagenic yet CrII is not [26]. Using the standard strains, their results indicate that CrVI reacts directly with bacterial DNA to cause both frame-shift and base-pair substitution mutations. Tindall et al. also found CrVI, K_2Cr_2O and K_2CrO_4, induced base pair substitution mutations in TA100 and *E. coli* 2 WP2uvrA [27]. When microsomal liver preparations are added to the Ames' system, CrVI mutagenicity was diminished due to metabolism [28].

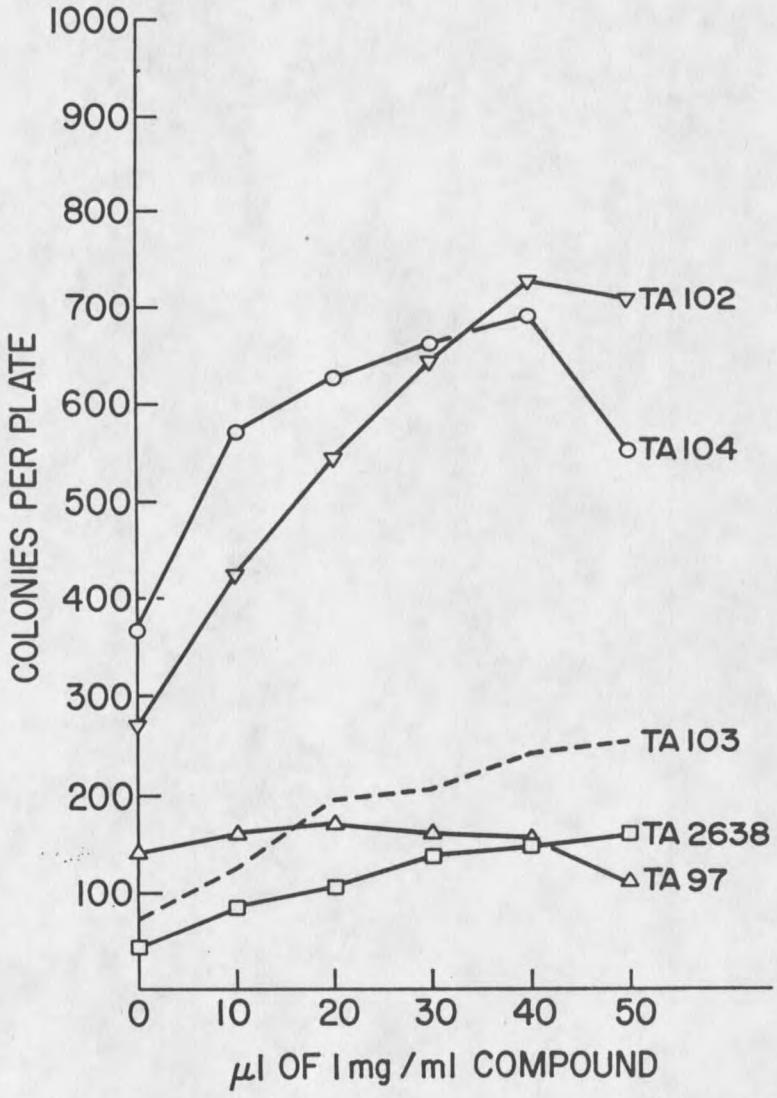


Figure 7. Dose-response curve with five strains of *S. typhimurium* with cis[$Cr(Bipy)_2 Cl_2$]-$Cl \cdot H_2O$, CrIII.

METHOD

The *Salmonella typhimurium* tester strains were a gift from Dr. Bruce Ames, University of California, Berkeley. See Tables 3 and 4 for the lists of chemicals and supplies used in this study.

Dose-Response Curves

Dose response curves were determined by using the Ames *Salmonella typhimurium* histidine reversion assay to produce his+ revertants. Nanomolar concentrations of direct-acting mutagens were added to the top agar along with the Salmonella strains and poured on to Nobles plates using the soft agar overlay technique as described by Ames [29]. Each concentration of mutagen was assayed in triplicate.

Millimolar concentrations of the platinum and chromium solutions were prepared in either sterile distilled water or dimethyl sulfoxide (DMSO). See Table 3 for list of metals used.

Tests were run in triplicate with the number of negative controls (spontaneous revertants) falling within 20% for each test with TA102 and TA103. A 20 μl amount of 5 mg/ml solution (or 0.1 mg) of the positive control Dexon, sodium[4-(dimethylamino)phenyl] diazenesulfonate, was added to the Nobles top agar. As Dexon induces both frameshift and basepair substitutions, all strains were assayed for their mutability with this compound.

The standard media were prepared according to the methods described by Ames [29]. See Appendix 1 for the components used for preparation.

Table 3. List of Compounds used for Mechanism and Dose-Response Studies.

1.	Cis $[Pt(NH_3)_2\ Cl_2]$ (Pt II)	Sigma Chemical Co.
2.	Cis $[Pt(NH_3)_2\ Cl_4]$ (Pt IV)	Chemistry Dept., MSU
3.	Cis $[Cr(Bipyridyl)_2\ F_2]\ F \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
4.	Cis $[Cr(Bipyridyl)_2\ Cl_2]\ Cl \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
5.	Cis $[Cr(Bipyridyl)_2\ Br_2]\ Br \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
6.	Cis $[Cr(Bipyridyl)_2\ I_2]\ I \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
7.	Cis $[Cr(Phenanthroline)_2\ F_2]\ F \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
8.	Cis $[Cr(Phenanthroline)_2\ Cl_2]\ Cl \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
9.	Cis $[Cr(Phenanthroline)_2\ Br_2]\ Br \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
10.	Cis $[Cr(Phenanthroline)_2\ I_2]\ I \cdot H_2O$ (CrIII)	Chemistry Dept., MSU
11.	$K_2Cr_2O_7$ (CrVI)	Chemistry Dept., MSU
12.	K_2CrO_4 (CrVI)	Chemistry Dept., MSU

Compounds 2 through 12 were synthesized by Mr. Dan Bancroft, Chemistry Department, Montana State University.
cis DDP = compound No. 1; (Bipy) = bipyridyl; (Phen) = Phenanthroline.

Table 4. Materials.

1.	N-(2-Thiazolyl)DL-alanine hydrate	Sigma Chemical Co.
2.	DL-1,2,4-Triazole-3-Alanine	Sigma Chemical Co.
3.	6 mm filter paper discs	Becton Dickman
4.	Dimethyl sulfoxide (DMSO) reagent grade	J. T. Baker Chemical Co.

Treated plates were incubated at 37 degrees C for 72 hours and visually assayed for individual colonies of his+ revertants. The presence of these his+ colonies, histidine independent, indicate either a base pair substitution, deletion (frameshift) mutation, or a mutation due to an extragenic suppressor has occurred in order for the bacteria to exist in the prototrophic state.

Mechanism Study

Individual colonies were picked from these plates with a sterile toothpick and grown up overnight (16 hours) in nutrient broth at 37 degrees C. Duplicate samples of each cultured his+ revertant were used to compare the microtiter method, developed in this work, with Levin's method of radially streaking the plates [9].

Each revertant colony was radially streaked onto a Nobles plate using a capillary tube. This method of streaking gave a more even growth pattern for TA102. Thiazolealanine (25 μl of 20 mg/ml sterile distilled water) or Triazolealanine (25 μl of 10 mg/ml) was applied to a 6 mm filter paper disc which was then placed in the center of the radially streaked plate. Plates were incubated at 37 degrees C for 24 hours.

For the comparative study of the plates with the microtiter assay, a duplicate sample of each cultured his+ revertant was diluted in Davis Minimal broth without casamino acids. A dilution of 1:500 was used for TA103 and 1:50 for TA102. Microtiter plates were used for an eight strain test (Figure 8), and 0.05 ml of Davis Minimal broth without caseamino acids was added to each well of the microtiter plate with the Cetus pro/pette.

Row 1 contained only bacteria and broth to serve as the cell control. Row 12 was not inoculated and served as a sterility control for the broth and analog. Row 2 received 0.05 ml of the histidine analog (either 10 mg/ml Thiazolealanine or 5 mg/ml Triazolealanine) with a 1-ml Tridak stepper. Serial dilutions were then performed automatically from row 2

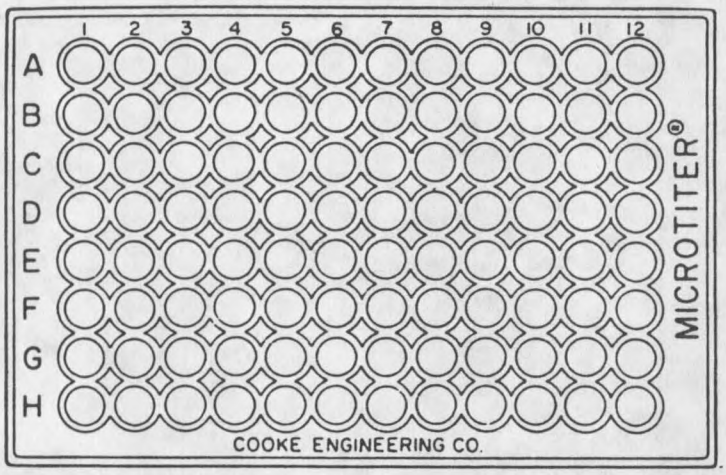


Figure 8. Microtiter plate. Row 1 = cell control, row 2 = sterility control, rows 2-11 = dilutions of analog, rows A-H = individual strains of his+ revertants.

through row 11 with the Cetus pro/pette. The final dilutions of the analog range from 1:1 in row 2 to 1:1024 in row 11. Row A through H each received one of the revertant strains of bacteria (His+).

RESULTS

There was an area of about 2 cm of inhibited growth on the streak plate test and a four to five-well difference with the microtiter assay between extragenic suppressors and point reversion mutations (Table 5; Figures 9 and 10).

Table 5. Correlation of Microtiter Plate and Pour Plates with Analog Dose.

Number Wells of Growth in Microtiter Plate	mm of Inhibited Growth of Revertants on Plate	
1	20	extragenic suppressor
1	18	extragenic suppressor
2	18	extragenic suppressor
10	0	deletion
1	24	extragenic suppressor
4	12	point revertant

Microtiter analog dose = 50 μl of 10 mg/ml. Thiazole.
Steak plate dose = 25 μl of 20 mg/ml. Thiazole.

With 3 to 6 base pairs deleted in the hisG428 position, the resulting enzyme becomes insensitive to feedback control and the his+ revertants grow up to the thiazole treated disc on the streaked plates and also show no sensitivity on the microtiter assay. Mutations with point reversions are sensitive to this analog and showed about 1 cm of inhibited growth on the plate and two to four wells difference on the microtiter plates (Figure 10; Table 6).

There is a positive correlation in results when the microtiter method is compared with radial streaking on plates (Table 6; Figures 9 and 10). By using the microtiter method as a phenotypic screen for deletion revertants, one can quantitate the number of His+ revertants formed by a small base pair deletion which removes part or all of the ochre mutation as well as base pair substitution at the ochre site and extragenic ochre suppressors.

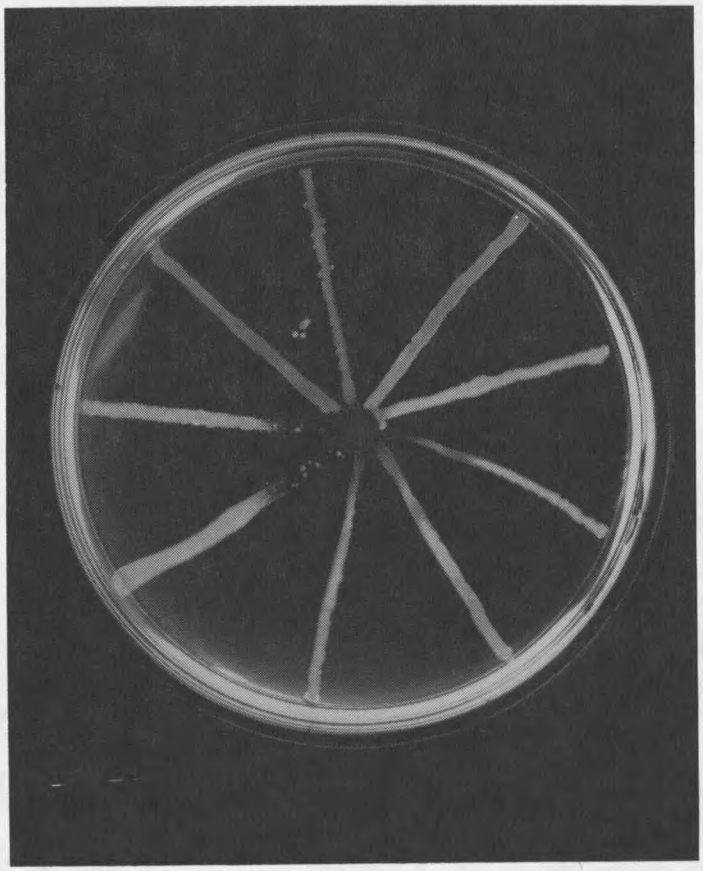


Figure 9. Streak plate showing Deletions, no inhibition; Point Reversions, sensitive to thiazolealanine; Extragenic suppressors, hypersensitive to thiazolealanine.

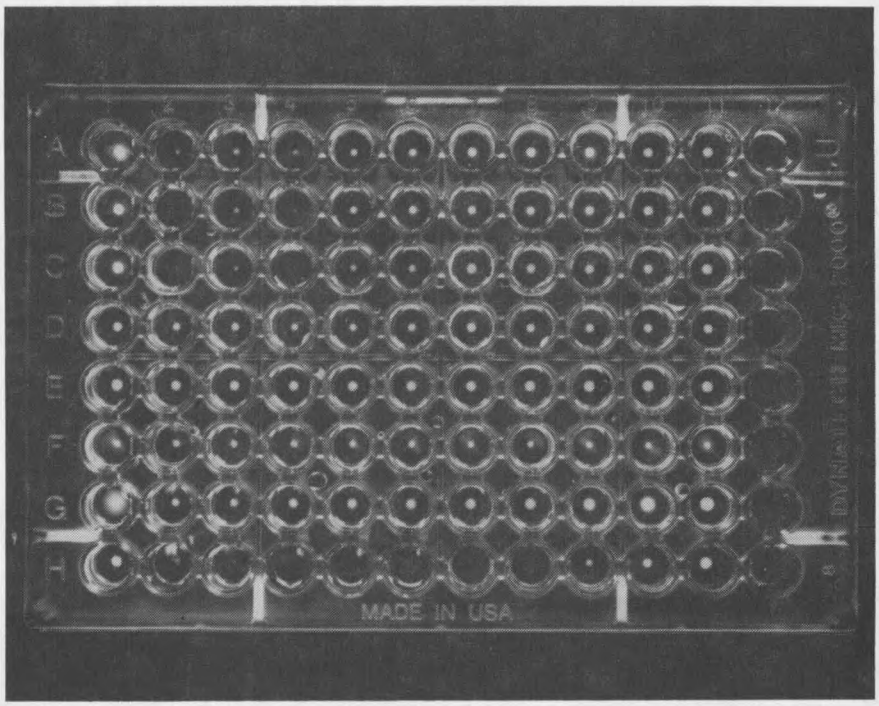


Figure 10. Microtiter plate. Row 2 received 50 μl of 10 mg/ml Thiazolealanine. A = TA2668 reversion; B = TA2669 reversion; C = TA2670 reversion; D = TA2671 deletion; E = TA2672 deletion; F = not inoculated; G = TA2674 deletion; H = E246 extragenic suppressor induced with cis[$Cr(Phen)_2\ I_2$].

Table 6. Levin Strains Treated with Two Analogs in Microtiter Study.

Strain Number	Wells of Growth in Microtiter Plates	
	Triazole	Thiazole
TA2668	7	7
TA2669	7	3
TA2670	7	8
TA2671	8	10
TA2672	8	10
TA2673	4	10
TA2674	8	10

Triazole = 5 mg/ml in well 2 with 50 μl dose in 50 ml broth.
Thiazole = 10 mg/ml in well 2 with 50 μl dose in 50 ml broth.

When using Levin's method of radial streaks, analog resistance is indicated by complete growth of the bacterial strain along the streak [9]. As seen in Table 6, there is also growth in wells 2-11 in the microtiter plate with the duplicate revertant strain showing 10 wells of growth for a deletion mutation. This is indicative of insensitivity to the histidine analog by a mutation due to deletion of base pairs. Little or no growth on the radial streak also correlates with growth in only the wells with the most lightly diluted analog concentration with 1 to 3 wells of growth. Base pair substitution mutations are sensitive to the analog, and extragenic suppressors show hypersensitivity.

DISCUSSION

Metal Mutagenicity

The dose response curves (Figures 7,11,12,13) show that all of the metals assayed produced a positive result in the Ames *Salmonella typhimurium* histidine reversion assay. Garland compared the mutagenic efficiency of a CrIII compound, cis[$Cr(bipy)_2\ Cl_2$], to a CrVI compound ($K_2Cr_2O_7$) in a GC reversion strain TA97 and with the AT reversion strains TA102, 103, 104, and 2638 (Figures 7,14) [30]. It was found that TA102 and 104 were the most sensitive strains for detecting mutagenicity in both CrIII and CrVI compounds. It appears that AT revertable sites are preferred by the CrIII species. If only GC revertable sites are available, CrVI is more efficient than CrIII. This implies that targeted mutagenesis is implemented as CrVI can damage DNA by a pathway not available to CrIII. Figures 11 and 12 show six CrIII species that exhibit a high degree of mutagenesis. The change in the cis halogenated ligands (F, Cl, Br, and I) did not appreciably affect the degree of mutagenicity of the CrIII compound. Figure 10 shows the mutagenicity of both PtII, cis DDP, and PtIV. The odd behavior of PtIV as seen in Figure 11 may be due to PtIV toxicity to TA102 at low nanomolar concentrations. The revertants per nanomole is listed in Table 7. Toxicity is implicated when the number of revertants per nanomole begin to decrease with the higher concentrations of metal ions.

Mechanisms of Metal Ion Mutagenesis

The implication of targeted mutagenesis is also supported by a study comparing the mechanism of spontaneous reversion and that which was induced by CrIII and CrVI

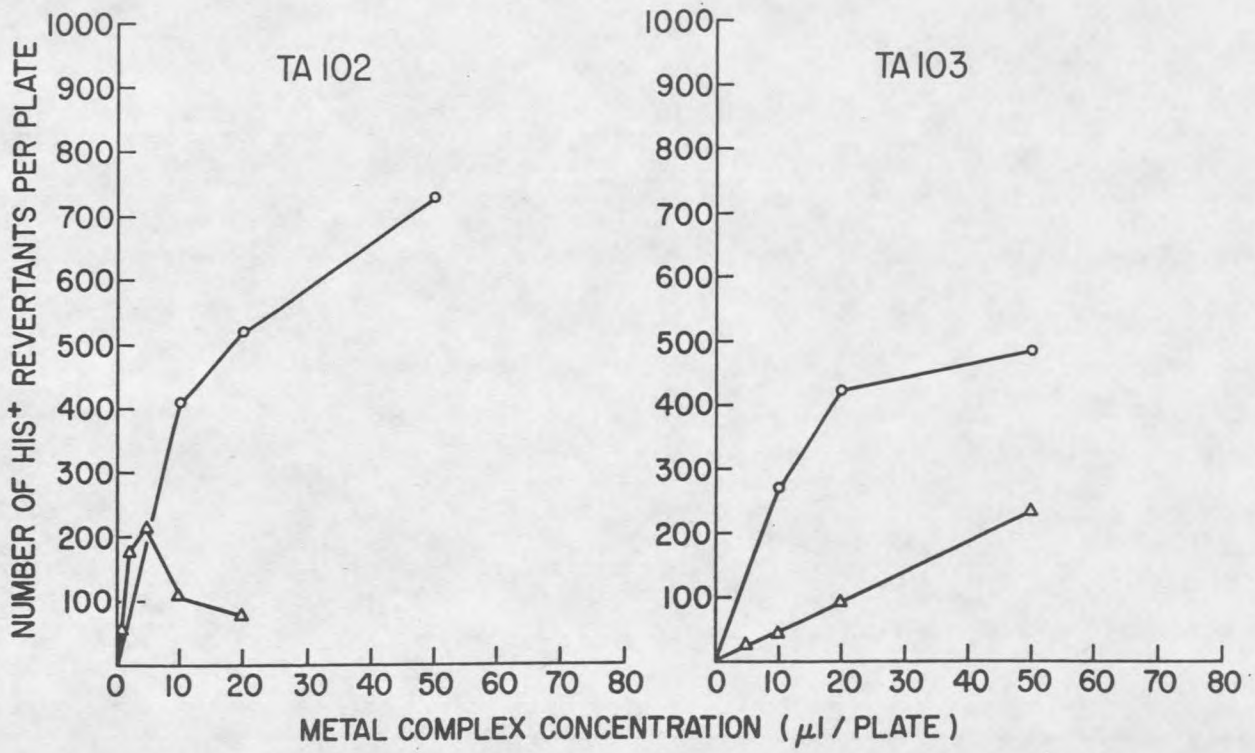


Figure 11. Dose-response curves for PtII and PtIV with both TA103 and TA102. ○ = PtII cis[Pt $(NH_3)_2$ Cl_2] ; △ = PtIV cis[Pt$(NH_3)_2$ Cl_2].

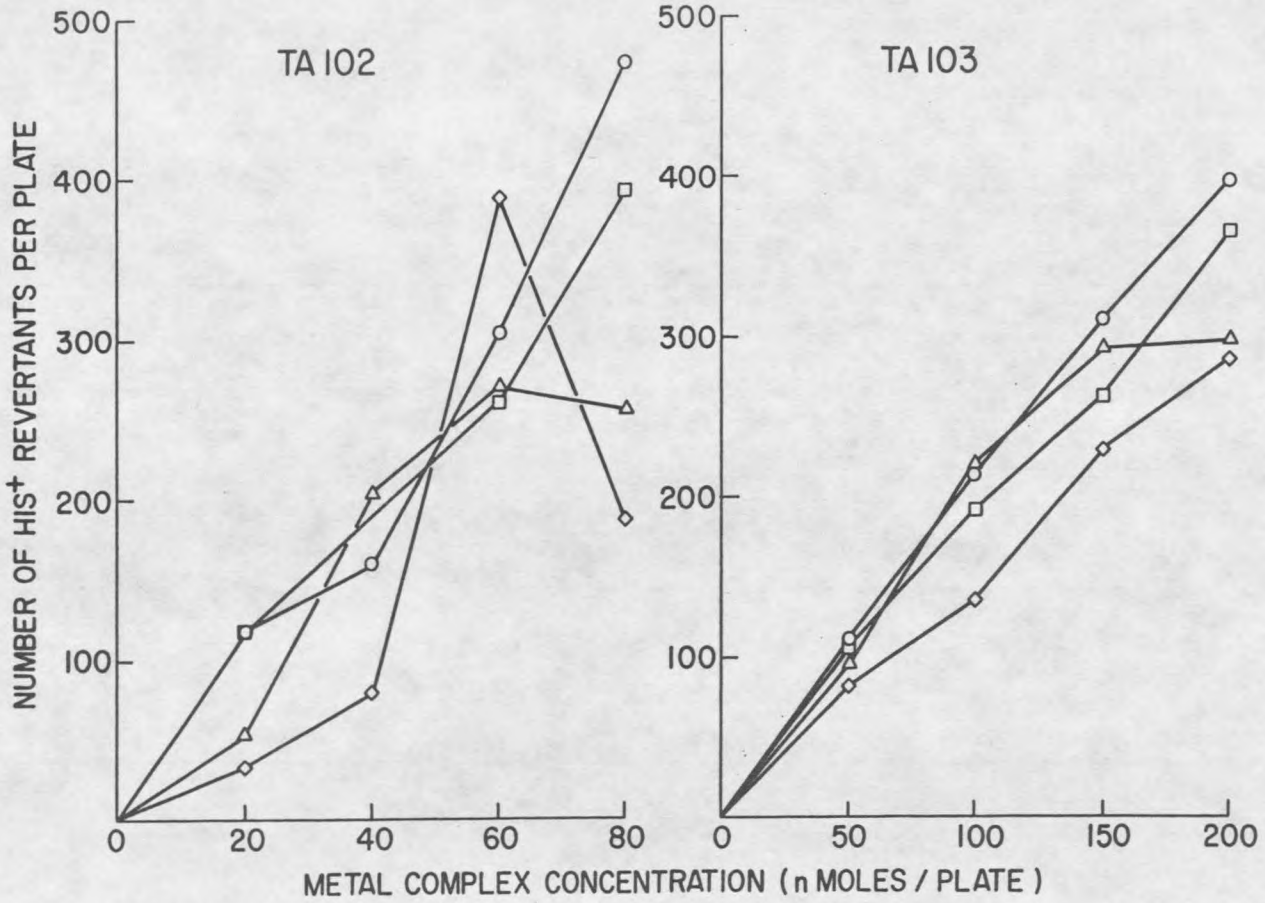


Figure 12. Dose-response curves using CrIII compounds with TA102 and TA103. △ = cis [Cr (Bipy)$_2$ F$_2$] ; ○ = cis [Cr(Bipy)$_2$ Br$_2$] ; □ = cis [Cr(Bipy)$_2$ Cl$_2$] ; ◇ = cis [Cr(Bipy)$_2$ I$_2$] .

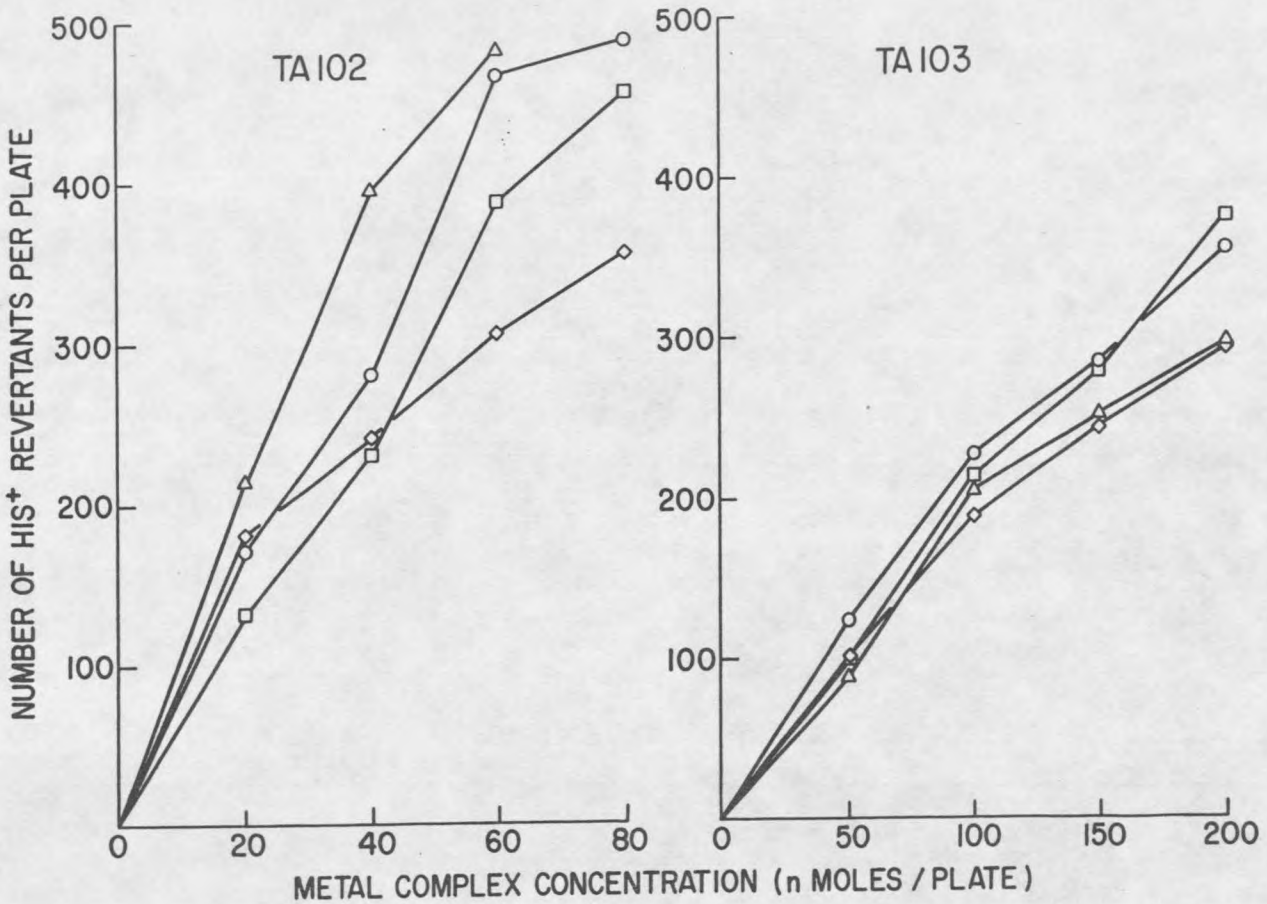


Figure 13. Dose-response curves for CrIII compounds with TA102 and TA103. △ = cis [Cr $(Phen)_2$ F_2]; ○ = cis [$Cr(Phen)_2$ Br_2]; □ = cis [$Cr(Phen)_2$ Cl_2]; ◇ = cis [$Cr(Phen)_2$ I_2].

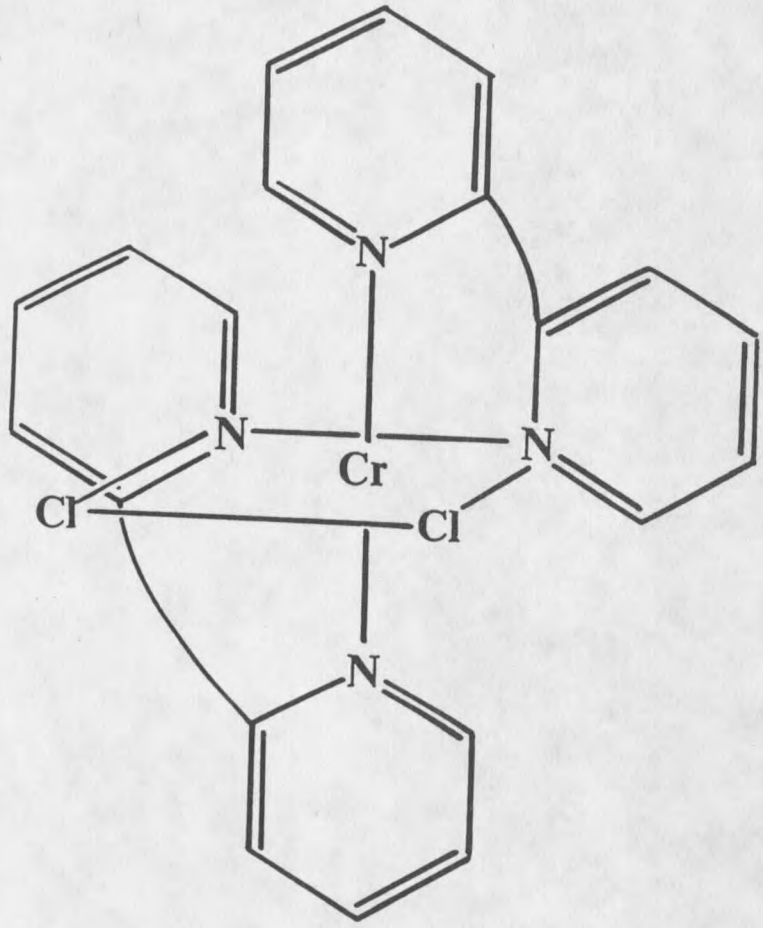


Figure 14. cis[Cr(Bipy)$_2$ Cl$_2$].

Table 7. Reactants per Nanomole for Chromium and Platinum Compounds.

Strain	Compound	Dose	Revertants/Nanomole
TA103	CrIII		
	239	200 nM	1.49
	240		1.83
	241		1.99
	242		1.43
	243		1.87
	244		1.78
	245		1.49
	246		1.45
TA102		80 nM	
	239		3.20
	240		4.91
	241		5.90
	242		2.35
	243		2.40
	244		2.50
	245		6.09
	246		4.46
TA103	PtIV		
		60 nM	5.6
TA102	PtIV	60 nM	9.3
TA103	PtII	60 nM	16.5
TA102	PtII	60 nM	22.5

using streaked plates (Fig. 15). With TA103, a more even distribution of extragenic suppressors (nine) and point reversions (fifteen) were observed. The his+ revertants from plates dosed with CrIII showed all to be extragenic suppressors. The CrVI his+ revertants are classified as point reversion with the exception of one extragenic suppressor. The fractions of mutagenic classes due to spontaneous reversions were subtracted from the induced mutagenic classes (Appendix II).

Again, when comparing the two species of Cr with strain TA103, $[Cr(Bipy)_2\ Cl_2]$, (CrIII), shows a higher percentage of extragenic suppressors than does K_2CrO_4, (CrVI) (Figure 15; Table 9). These two studies were done using different plates for streaking. The former tests were run on Difco VB plates whereas the latter used Nobles for growing revertants.

The mechanistic study for $cis[Pt(NH_3)_2\ Cl_2]$, PtII, and $cis[Pt(NH_3)_2\ Cl_4]$, PtIV with TA103 show the strongest case for targeted mutagenesis. There is a striking difference in the change of the fraction of extragenic suppressors and point reversions as compared with those of the uninduced TA103 his+ revertants (Table 3; Figure 16). The tests were repeated three times with a total of 144 revertants. Nearly 100% of the mutations resulting from point reversions whereas there were only 37% point reversions in the uninduced bacteria.

TA102 showed more variability between tests with a slight increase in the number of extragenic suppressors per plate and a decrease in the total of point reversions per plate (Table 8).

The mechanisms of mutation of the CrIII compounds show a higher percentage of point reversions than is found in the uninduced or spontaneous revertants of TA103. The number of deletions remain low as expected with the hisG428 mutation on the chromosome rather than on a multicopy plasmid as found in TA102. It is also interesting to note

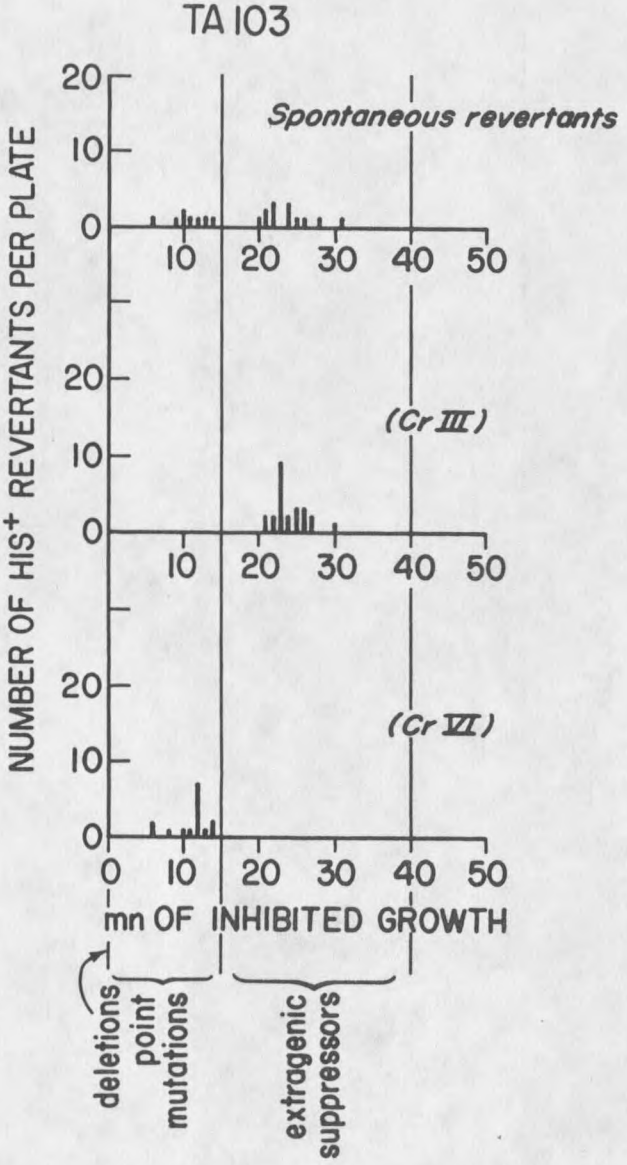
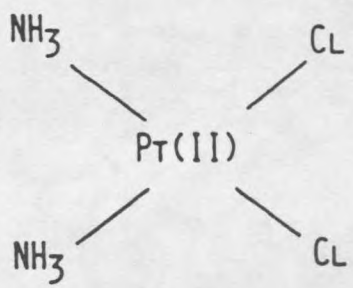


Figure 15. Mechanism study comparing CrIII, cis $[Cr(Bipy)_2\ Cl_2]$ and CrVI, K_2CrO_4.



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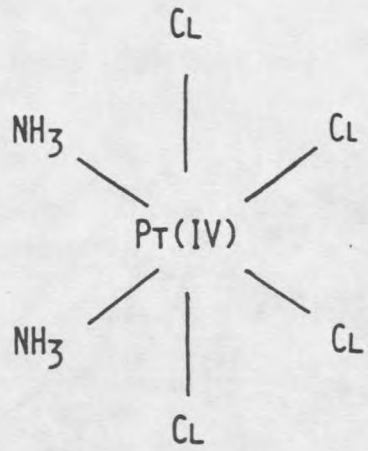

II

Figure 16. I = cis[Pt(NH_3)$_2$ Cl_2] ; II = cis [Pt(NH_3)$_2$ Cl_2].

Table 8. A Comparison of Mechanisms of Mutagenesis with PtII and PtIV.

	Uninduced	PtII	PtIV
	Fraction of Deletions, Point Reversions, and Extragenic Suppressors		
TA102			
Reversions	23%	19%	6%
Extragenic	40%	49%	45%
Deletions	37%	32%	49%
Number of revertants	48	96	96
TA103			
Reversions	37%	97%	94%
Extragenic	59%	1%	5%
Deletions	4%	2%	1%
Number of revertants	48	144	144

that the two chlorinated CrIII compounds showed the same ratios of mutation classifications; R = 91%, E = 9%, D = 0% (Table 9).

With TA102 there are generally more deletions found in Cr induced mutations than the uninduced (Table 10). There is also a general trend toward fewer point reversions in the induced mutations rather than in the uninduced mutations.

Table 9. A Comparison of Mechanisms of Mutagenesis with CrIII and CrIV.

	Spontaneous Revertants	Cr239 Cr/Bipy/F	Cr240 Cr/Bipy/Cl	Cr241 Cr/Bipy/Br	Cr242 Cr/Bipy/I
	Fraction of Deletions, Point Reversions, and Extragenic Suppressors				
TA103					
Reversions	40%	58%	91%	76%	63%
Extragenic	56%	42%	9%	24%	37%
Deletions	4%	0%	0%	0%	0%
Each test consists of 48 revertants					
	CrVI $K_2 Cr_2 O_7$	Cr243 Cr/Phen/F	Cr244 Cr/Phen/Cl		Cr245 Cr/Phen/Br
TA103					
Reversions	55%	73%	91%		100%
Extragenic	40%	27%	9%		0%
Deletions	7%	0%	0%		0%
Each test consists of 48 revertants each					

Table 10. A Comparison of Mechanisms of Mutagenesis with CrIII and CrVI.

	Spontaneous Revertants	Cr239 Cr/Bipy/F	Cr240 Cr/Bipy/Cl	Cr241 Cr/Bipy/Br	Cr242 Cr/Bipy/I
	Fraction of Deletions, Point Reversions, and Extragenic Suppressors				
TA102					
Reversions	23%	26%	0%	0%	12%
Extragenic	40%	45%	43%	48%	42%
Deletions	37%	25%	57%	52%	46%
Each test consists of 48 revertants each					
	Spontaneous Revertants	Cr243 Cr/Phen/F	Cr244 Cr/Phen/Cl	Cr245 Cr/Phen/Br	Cr246 Cr/Phen/I
TA102					
Reversions	23%	30%	20%	7%	0%
Extragenic	40%	41%	20%	33%	33%
Deletions	37%	30%	60%	60%	37%
Each test consists of 48 revertants each					

SUMMARY

In summary, the recently derived ochre hisG428 strains, TA102, 103, 104, and 2638, are particularly useful in the analysis of the mutagenic activity of substitutionally inert transition metal complexes as well as the CrVI compound, potassium dichromate. Because of the fortuitous location of the hisG428 mutation in the structural G gene, the his+ revertants differentially respond to feedback inhibition of encoded enzyme. Accordingly, revertants will either retain or lose the feedback inhibition site thus allowing different categories of revertants to have a differential sensitivity to the histidine analog, thiazolealanine. The histidine analog allows the identification of the induced mutations into three categories: (a) small deletions, (b) base pair mutations, and (c) extragenic suppressors with TA102 while induced mutations in TA103 only result in categories b and c.

We have developed a microtiter suspension assay system that can quantitatively relate analog concentration to growth inhibition of the particular category of mutant. This research indicates that dichromate (CrVI) induces mainly point revertants while CrIII, $[Cr(bipy)_2\ Cl_2]$, induced predominantly extragenic suppressors in TA103, PtII, cis $[Pt(NH_3)_2\ Cl_2]$, induced about equal numbers of extragenic suppressors and point revertants while PtIV, cis $[Pt(NH_3)_2\ Cl_2]$ induced point revertants in TA103. PtII and PtIV induced about an even distribution of all three types in TA102.

LITERATURE CITED

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1. Legator, M. S., and W. S. Flam. 1973. Ann. Rev. Biochem. Vol. 42: 683-708.
2. McCann, J. Hospital Practice. Sept. 1983: 73-85.
3. McCann, J. et al. 1975. Proc. Nat. Acad. Sci. USA. Vol. 72, No. 12: 5135-5139.
4. Ames, B. N. 1979. Science. Vol. 204: 587-591.
5. Miller, E. C., and J. A. Miller. 1971. *Chemical mutagens: Principles and methods for their detection*. Ed. Hollander, A. (Plenum Press, New York). Vol. I: 83-119.
6. Johnston, M. H., W. M. Barnes, F. Chumley, L. Bossi, and H. R. Roth. 1980. Proc. Nat. Acad. Sci. USA. Vol. 77. No. 1: 508-512. Genetics.
7. Miller, J. H., and S. W. Reznikoff. 1980. The Operon, Cold Spring Harbor: 273-274.
8. Levin, D. E. et al. 1982. Proc. Nat. Acad. Sci. USA. Vol. 79: 7445-7449. Genetics.
9. Levin, D. E. et al. 1984. Proc. Nat. Acad. Sci. USA. Vol. 81: 4457-4461. Genetics.
10. Albertini, A., M. Hofer, M. P. Calos, and J. H. Miller. 1982. Cell. Vol. 29: 319-328.
11. Streisinger, G., Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, and M. Inouye. 1966. *Frameshift mutations and the genetic code*. Cold Spring Harbor Symposium, Quant. Biol. Vol. 31: 77-84.
12. Coulondre, C., and J. H. Miller. 1977. J. Mol. Biol. Vol. 117: 577-606.
13. Miller, J. H. 1982. Cell. Vol. 31: 5-7.
14. Walker, G. C. March 1984. Microbiological Reviews: 60-93.
15. Tullius, T. D., and S. J. Lippard. 1982. Proc. Nat. Acad. Sci. USA. Vol. 79: 3489-3492. Biochemistry.
16. Andersen, K. S. 1979. Mutation Research. Vol. 17: 209-214.
17. Maxam, A. M., and W. Gilbert. 1980. Methods of Enzymol. Vol. 65: 499-560.
18. Krugh, T. R., J. W. Hook, S. Lin, and F. M. Dhen. 1979. *Stereodynamics of Molecular Systems*. Ed. Sarma, R. H. Pergamon, New York: 423-435.

19. Wang, A. H. J., G. J. Quigley, F. S. Kolpak, G. vanderMaul, J. H. vanBoom, and A. Rich. 1981. Science Vol. 211: 171-176.

20. Cohen, G. L., W. R. Baur, J. K. Barton, and S. J. Lippard. 1974. Science. Vol. 203: 104.

21. Barton, J. K., and S. J. Lippard. 1980. Nucleic acid-metal ion interaction. Ed. Spiro, T. G. (Wiley, New York): 31-113.

22. Lippard, S. J. 1982. Science. Vol. 218, No. 4577: 1075-1082.

23. Sarma, B. D., S. K. Daley, and R. K. Elerspuru. 1983. Chem-Biol. Interaction. Vol. 46: 219-232.

24. Bennicelli, C. et al. 1983. Mutation Research. Vol. 122: 1-5.

25. Langerwerf, H. A. B., and W. M. T. Jongen. 1985. Ecotoxicology and Environmental Safety. Vol. 9: 92-100.

26. Pertilli, F. L., and S. DeFlora. 1977. Appl. Environ. Microbiol. Vol. 33: 805.

27. Tindall, K. R., G. W. Warren, and P. D. Skaar. 1977. Abstracts, 8th Annual Meeting. Environmental Mutagen Society, Colorado Springs, Colorado.

28. Lofroth, G. 1978. Naturwissenschafter. Vol. 65: 297.

29. Ames, B. N., J. McCann, and E. Yamasaki. 1975. Mutation Res. Vol. 31: 347-364.

30. Garland, J., G. Warren, and S. J. Rogers. 1985. *Comparative Mutagenic Efficiency of CrVI and CrIII in Reverting GC vs AT in the Salmonella Strains TA97, 100, 102, 103, 104, 2638.* Environmental Mutagen Society meeting, Las Vegas, Nev.

APPENDICES

APPENDIX I

APPENDIX I

Nobles Agar (Plates)

In 2,000 ml flask

710 mls	D.D. H_2O
12 g	Noble agar
20 g	dextrose

In 1000 ml flask

40 mls	25x VB salts
250 mls	D.D. H_2O
10 mls	mineral solution

Autoclave 20 minutes in separate flasks. Let cool 10 minutes then add small flask to large flask using sterile technique. Shake well.

Nobles Top Layer

.6 g	Nobles agar
.5 g	NaCl
99.9 mls	double distilled H_2O
5.0 mls	0.5 mM histidine (use sterile technique)
5.0 mls	0.5 mM biotin (use sterile technique)
0.1 mls	mineral solution

Use small screw top bottles. Autoclave 20 minutes. Add biotin and histidine steriley. Shake well.

Difco Nutrient Broth

0.5 g	NaCl
0.8 g	nutrient broth
99.9 mls	double distilled H_2O
0.1 mls	mineral solution

Use small screw-top bottles. Shake well. Autoclave 20 minutes.

Davis Minimal without Caseamino Acids

1.0 gm	dextrose
0.8 gm	DM broth
98.9 ml	double distilled H_2O

1.0 ml	minerals
0.1 ml	0.5 mM biotin

Shake well. Must be completely dissolved. Autoclave 20 minutes.

Vogel Bonner Salts 25x

1000.0 mls	double distilled H_2O
5.0 gm	$MgSO_4 \cdot 7HO$)
50.0 gm	citric acid$\cdot H_2O$
250.0 gm	K_2HPO_4 (anhydrous dibasic)
87.5 gm	$NaNH_4PO_4 \cdot 4H_2O$

Using a volumetric flask (1000 ml) fill ½ full with H_2O. Add compounds one at a time in the above order dissolving one completely before adding the next. After all is added bring volume to 1000 ml mark, separate into two screw-top flasks and autoclave 20 minutes.

0.5 mM	histidine
7.8 gm	L-histidine
100 mls	double distilled H_2O

Autoclave 20 minutes

0.5 mM	biotin
12.2 mg	biotin
100 ml	double distilled H_2O

Autoclave 20 minutes

Mineral Solution

0.05 gm	$CaCl_2$
0.025 gm	$MnCl_2$
0.015 gm	$ZnCl_2$
0.15 gm	$FeSO_4$
500 mls	double distilled H_2O

Autoclave 20 minutes in screw-top flask.

APPENDIX II

APPENDIX II

Method for Calculations of Fractions of Mutations Attributed to Induction

In order to adjust our raw data for the number of revertants which may be due to spontaneous mutations, the following method was employed.

column 1 = type of induction
column 2 = the number of revertants per plate
column 3 = raw data R = point reversions
E = extragenic suppressors
D = deletions
column 4 = the fraction of the total number of revertants (col. 2) due to spontaneous reversion; the spontaneous divided by the induced.
column 5 = the theoretical number of streaked revertants due to spontaneous mutation. (col. 4 multiplied by the total number in col. 3)
column 6 = the number of each class of mutation due to spontaneous reversion. (col. 5 multiplied by the fraction of each class of mutation from the spontaneous revertants)
column 7 = the adjusted number of revertants due to induction. (col. 3 subtracted from col. 6)
column 8 = the fraction of adjusted induced revertants within the total population of the adjusted number of mutations. (col. 7 divided by total "R E Ds" from col. 7)

Example:

Ta103

Compound 1	No. Revertants 2	Raw "REDs" 3	% Uninduced 4	% Due to Uninduced 5	No. Due to Uninduced 6	Adjusted Induced 7	"REDs" 8
Uninduced	139	R 19					19/48=40%
		E 27					27/48=56%
		D 2					2/48=04%
Cr 239	402	R 25	139/402= .35	.35X48= 16.8	17X.40=7	25-7=18	18/31 =58%
		E 22			17X.56=9	22-9=13	13/31 =42%
		D 1			17X.04=1	1-1=0	0%

Vieux, B. A.
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