A STUDY OF
THE GENETIC HETEROGENEITY
IN ROBERTS SYNDROME

by

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ABSTRACT

Roberts syndrome (RS) is a rare, recessive condition characterized by growth retardation, developmental delay and tetraphocomelia. Some RS patients (RS+), but not others (RS-), exhibit a "puffing" of the constitutive heterochromatic regions of their chromosomes (the "RS effect"). Cells from RS+ patients also show cellular hypersensitivity to DNA crosslinking agents such as mitomycin C (MMC). In the present study, correction of both the RS effect and MMC hypersensitivity in somatic cell hybrids between RS+ and normal lymphoblastoid cells (LCLs) supported the hypothesis of an association between the RS effect and mutagen sensitivity in RS+ cells. Somatic cell hybrids between two RS+ LCLs derived from patients with diverse ethnic backgrounds exhibited both the RS effect and MMC hypersensitivity indicating that these patients represent a single complementation group. Somatic cell hybrids between one of the RS+ and two different RS- LCLs demonstrated complete complementation of both the RS effect and MMC hypersensitivity. These findings suggest that RS+ and RS- patients belong to different complementation groups.

Fanconi anaemia (FA) is another rare, recessive disorder characterized by growth retardation, developmental

delay, limb abnormalities and progressive pancytopenia. Cells from FA patients exhibit both chromosomal and cellular hypersensitivity to DNA crosslinking agents. A study of the sensitivity and mutability of various LCLs by ethyl methanesulphonate (EMS) indicated an increased cellular sensitivity and decreased mutability of RS+ LCLs relative to control LCLs. RS- LCLs did not exhibit these phenomena. One FA LCL from complementation group A showed a slightly increased cellular sensitivity but normal mutability. These results suggest hypomutability by EMS may be associated with the RS effect and MMC hypersensitivity.

Somatic cell hybrids were made between one of the RS+ LCLs and an LCL from each of the four known FA complementation groups. Hybrids were examined for correction of MMC hypersensitivity, the RS effect and diepoxybutane-induced chromosome aberrations. Complementation was observed in hybrids with FA LCLs from complementation groups A, B and D but incomplete correction of chromosomal and cellular sensitivities to crosslinking agents in RS+ x FA C hybrids suggested a genetic association.

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LIST OF ABBREVIATIONS

ara C	cytosine arabinoside
AT	ataxia telangiectasia
BrdUrd	5-bromodeoxyuridine
CHO	Chinese hamster ovary
CREST	calcinosis, Raynaud's phenomenon, esophageal dismotility, sclerodactyly, telangiectasia
C-RI	co-recessive inheritance
D_{10}	the dose at which 10% cellular survival is observed
DEB	diepoxybutane
DMCC	decarbamoyl mitomycin C
DRF	dose reduction factor
EC_{50}	the dose at which 50% relative cloning efficiency is observed
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulphonate
FA	Fanconi anaemia
FBS	fetal bovine serum
HAT	hypoxanthine-aminopterin-thymidine
HPRT	hypoxanthine-guanine phosphoribosyl transferase
IFAR	International Fanconi Anaemia Registry
IL-6	interleukin-6
LCL	lymphoblastoid cell line
MFRF	mutant frequency reduction factor

LIST OF ABBREVIATIONS (continued)

MMC	mitomycin C
8-MOP	8-methoxypsoralen
PBS	phosphate buffered saline
pI	isoelectric point
R-52	tetraetansulfonil-d-mannit
RS	Roberts syndrome
RS+	Roberts syndrome with the constitutive heterochromatin abnormality
RS-	Roberts syndrome without the constitutive heterochromatin abnormality
SCE	sister chromatid exchange
sd	standard deviation
6-TG	6-thioguanine
TMP	4,5',8-trimethylpsoralen
Tris	Tris (hydroxymethyl) aminomethane
VNTR	variable number tandem repeat
XP	xeroderma pigmentosum

1. INTRODUCTION

1.1 Roberts Syndrome

Roberts syndrome (RS) was first described by Dr. J.D. Roberts in 1919 in "a child with double cleft of lip and palate, protrusion of the intermaxillary portion of the upper jaw and imperfect development of the bones of the four extremities" (Roberts, 1919). Despite other reports of patients with a similar combination of abnormalities (O'Brien and Mustard, 1921), RS was not recognized as a syndrome until 1966 (Appelt et al.). Today, RS is described as a rare, heritable condition characterized primarily by pre- and post-natal growth retardation, developmental delay and reduction abnormalities of all four limbs. In addition, facial abnormalities (including cleft palate with or without cleft lip and mid-facial hemangioma), renal defects, cardiac anomalies, cranial defects and variable mental retardation have been reported in some patients (O'Brien and Mustard, 1921; Herrmann et al., 1969; Judge, 1973; Freeman et al., 1974; Grosse et al., 1975; Mann et al., 1982; Grundy et al., 1988).

A wide range of clinical presentations have been reported in RS. While the majority of patients die in the

perinatal period as a result of multiple, severe abnormalities, patients with very mild clinical manifestations have also been described (Herrmann and Opitz, 1977; Petrinelli et al., 1984; Stanley et al., 1988). A number of different terms have been used to describe RS in the literature. "SC-phocomelia" (SC refers to the initials of two families studied) was used to describe more mildly affected patients (Herrmann and Opitz, 1977). The terms "Appelt-Gerken-Lenz" syndrome and "pseudothalidomide" syndrome have also been used in the literature to describe more severely and less severely affected patients, respectively (Freeman et al., 1974; Herrmann and Opitz, 1977). Observations of both RS and SC-phocomelia occurring in single sibships led to the conclusion that these two conditions represent a single, heterogeneous genetic entity (Herrmann et al., 1969; Levy et al., 1972; Judge, 1973; Kucheria et al., 1976; Zergollern and Hitrec, 1976, 1982; Qazi et al., 1979; Fryns et al., 1980, Mann et al., 1982; Romke et al., 1987; Gil et al., 1988).

Pedigree analysis of RS families has led to its designation as an autosomal recessive disorder (McKusick, 1990, 268300). In all cases, parents of affected children were unaffected and consanguinity has been reported in a number of cases (O'Brien and Mustard, 1921; Stroer, 1939; Mietens and Weber, 1966; Levy et al., 1972; Judge, 1973; Fitzsimmons, 1980; Mann et al., 1982; Zergollern and Hitrec,

1982; da Silva and Bezerra, 1982; Wenger et al., 1988; Lin et al., 1988). In one case, concordance between identical twins was reported (Fryns et al., 1980). No predominance of RS in either sex has been noted.

The first indication of abnormal cytogenetic findings in an RS patient was described as a consistently abnormal appearance of the centromeric regions with those of the acrocentric chromosomes being particularly noticeable (Judge, 1973). No explanation nor clinical importance was suggested for these findings. In 1974, Freeman and co-workers described an unusual cytogenetic finding in another RS patient (Freeman et al., 1974). The sister chromatids exhibited a localized separation usually but not exclusively at or near the centromeres. Folic acid deficiency was suggested as a possible explanation of this unusual chromosome morphology although no biochemical evidence of this could be found. Five years later, two papers were published describing a similar phenomenon in other RS patients (Tomkins et al., 1979; German, 1979). The "RS effect" as it is now known, is an abnormality of the constitutive heterochromatin that is best described as a "puffing" of the paracentromeric regions, the nucleolar organizing regions and the long arm of the Y chromosome (Tomkins et al., 1979; German, 1979; Louie and German, 1981). This phenomenon has been observed in a number of cell types from RS patients including fibroblasts,

lymphocytes and lymphoblasts (Tomkins et al., 1979; Jabs et al., 1991).

Interestingly, not all RS patients exhibit the RS effect; some patients, although clinically indistinguishable from others, exhibit normal chromosome morphology (Tomkins and Sisken, 1984; Grundy et al., 1988; Fryns et al., 1987). To distinguish between the two subgroups of patients, those exhibiting the RS effect have been designated RS+ while those without it as RS- (Burns and Tomkins, 1989). Given that prior to the last decade, RS patients were not studied for the presence of the RS effect, the ratio of RS+ to RS- patients is unclear. An estimate of 50% of patients being RS+ was made based on a review of the literature in 1984 (Tomkins and Sisken, 1984) More recently, 80% of patients were said to be RS+ but the basis for this estimate was not stated (Jabs et al., 1991). Although no obvious clinical differences exist between RS+ and RS- patients, two RS+ patients have been reported to have developed malignancies (Parry et al., 1986; Wenger et al., 1988). Given that fewer than 100 cases of RS have been reported and assuming that approximately 50% of these cases were RS+, this may represent an increased risk of malignancy to RS+ but not RS- patients.

The underlying cause of the RS effect has not been determined. Initially, the possibility of vitamin deficiency in RS+ patients was suggested (Freeman et al.,

1974). Work with RS+ fibroblasts, however, revealed that supplementing culture medium vitamin B_{12} did not restore normal chromosome morphology (Gunby, 1986).

Given that the constitutive heterochromatic regions affected by the RS effect appear to be de-condensed, it was hypothesized that drugs which affect normal centromere morphology such as cytosine arabinoside (ara C) and 5-azacytidine may produce a similar phenomenon in normal cells. While it was found that treatment of lymphocytes with ara C did significantly increase the frequency of chromosome breaks, no phenomenon resembling the RS effect was observed. Thus, inhibition of DNA synthesis through alteration of nucleoside pools is not sufficient to explain the RS effect. Similarly, treatment of lymphocytes with 5-azacytidine was found to result in a stretching of the constitutive heterochromatin around centromeres that remained tightly apposed. This is a different morphology from that seen in RS+ cells thus indicating that simple demethylation of heterochromatin was not sufficient to explain the RS effect (Gunby, 1986). In addition, it has recently been demonstrated that, while the decondensed centromeric regions produced by 5-azacytidine treatment did not exhibit C-like banding when digested with the restriction enzyme *AluI*, the decondensed paracentromeric regions of RS+ patients did show normal *AluI* banding (Arn and Jabs, 1990). This suggests that the abnormal appearance of the constitutive

heterochromatin in RS+ chromosomes is not due to a decondensation analogous to that produced by 5-azacytidine treatment. Recently, a significant hypomethylation of the constitutive heterochromatin of RS+ patients compared to that of control and RS- patients has been demonstrated (Harrison and Tomkins, 1991a). However, there was also a significant effect of *in vitro* aging, so this abnormality was not thought to be the primary defect in RS+ cells.

The proteinaceous composition of the centromeres of RS+ patients has been examined using antibodies to centromere proteins (CENP-A, -B and -C) and antibodies from patients with CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) which include antikinetochore and anticentromere antibodies (Jabs *et al*., 1991; Maserati *et al*., 1991). In neither case was any abnormal protein composition noted. Electron microscopic examination of the kinetochores of RS+ patients has revealed a normal trilaminar kinetochore structure (Jabs *et al*., 1991).

The RS effect, therefore, is a characteristic abnormality of the constitutive heterochromatin that is observed in all cell types examined to date in a proportion of RS patients. Despite the decondensed appearance of the heterochromatin, the RS effect does not appear to be due the a deficiency of vitamin B_{12} nor does it appear to be analogous to the phenomena observed in cells treated with

ara C or 5-azacytidine. Similarly, a significant decrease in methylation levels in RS+ cells is not considered to be the primary cause of the RS effect. Finally, neither the proteinaceous composition of the centromeric regions nor the kinetochore structure of RS+ is abnormal. Thus, while a number of possible causes of the RS effect have been ruled out, the basis of this abnormality remains unknown.

Attempts to correct the RS effect have been made. Co-cultivation of RS+ fibroblasts or lymphocytes with either normal human or Chinese hamster ovary (CHO) cells failed to correct the RS effect (Knoll et al., 1983; Wertelecki et al., 1983; Petrinelli et al., 1984; Krassikoff et al., 1986). These observations suggested that the RS effect is not caused by the lack of a diffusible factor. Somatic cell hybridization experiments between RS+ and normal human or rodent cells have proven more successful in achieving correction of the RS effect (Krassikoff et al., 1986; Knoll and Ray, 1986; Gunby et al., 1987). Hybridization of RS+ fibroblasts with normal fibroblasts was shown to result in correction of the RS effect in 95% and 92% of hybrid cells in two separate experiments (Gunby et al., 1987). Similarly, correction of the RS effect in most RS chromosomes was reported in lymphoblast-fibroblast and fibroblast-fibroblast hybrids between RS+ and normal cells (Knoll and Ray, 1986). Finally, interspecies hybrids between RS+ fibroblasts and Chinese hamster lung cells also

exhibited correction of the RS effect (Krassikoff *et al.*, 1986). These studies indicated that the RS effect is caused by a lack or deficiency of a gene product in the RS+ cells.

The presence of the RS effect in the cells of some RS patients led researchers to investigate *in vitro* growth characteristics of fibroblasts from RS+ patients compared to those of control fibroblasts (Tomkins and Sisken, 1984). It was found that RS+ fibroblasts have significantly decreased plating efficiencies, colony forming abilities and cell numbers at confluence than do control cells. In addition, time-lapse cinemicrography revealed that while 98.2% of normal cells have metaphase durations of less than 18 minutes and 100% of these cells completed cell division in the course of the experiment, more than 30% of RS+ fibroblasts had metaphase durations of greater than 21 minutes and nearly 20% took longer than 36 minutes or failed to complete mitosis all together. These results were recently confirmed in another study which found that 12/29 (41.4%) RS+ fibroblasts studied failed to complete mitosis over the course of a 96 hour experiment (Jabs *et al.*, 1991). Abnormal cleavage furrows in cytokinesis were also observed in RS+ cells in the original study (Tomkins and Sisken, 1984). It was suggested that these abnormal cellular growth characteristics may explain some of the clinical manifestations of RS such as growth retardation and developmental abnormalities (*ibid.*).

Recently, further studies into the mitotic behaviour of RS+ cells have been undertaken (Jabs _et al_., 1991; Harrison and Tomkins, 1991b). Although no evidence for an increased rate of spontaneous chromosome breakage was found in the RS+ cells, a high frequency of aneuploidy was noted in lymphocytes, lymphoblasts and fibroblasts with random chromosome loss being more common than chromosome gain. No relationship between chromosome number and the presence of the RS effect was noted. Examination of interphase nuclei revealed that nuclear blebs and lobules and micronuclei were found five- to ten-times more frequently in RS+ cells. It was suggested that these abnormal nuclear morphologies were the result of "lagging" chromosomes during anaphase as such chromosomes were observed in 8% of cells in _in situ_ chromosome preparations. Less than 1% of RS-, RS+ obligate heterozygote and control cells exhibited lagging chromosomes (Jabs _et al_., 1991). All of these abnormalities of mitosis led the authors to suggest that RS+ patients represent a mitotic mutant.

It has been shown using 5-bromodeoxyuridine (BrdUrd) incorporation that RS+ fibroblasts synthesize DNA at a slower rate than control fibroblasts (Gani _et al_., 1984). The replication programme of RS+ constitutive heterochromatin during S phase was compared to that of control constitutive heterochromatin (Harrison and Tomkins, 1991b). While the S phase subphase distribution of RS+

lymphoblastoid cells was not found to be significantly different from control lymphoblastoid cells, the RS+ constitutive heterochromatin was found to replicate later in S phase compared to the control constitutive heterochromatin. It was suggested that this delay in constitutive heterochromatin replication could affect proper chromatin assembly thus resulting in the abnormal appearance of the constitutive heterochromatic regions in RS+ cells. Alternatively, the abnormal heterochromatin structure could cause the delay in replication (Harrison and Tomkins, 1991b).

Abnormal alleles of the _mus_-101 gene in _Drosophila melanogaster_ have been shown to be associated with both abnormal heterochromatin condensation and mutagen hypersensitivity (Gatti _et al_., 1983). These authors identified a temperature-sensitive mutant at this locus that causes abnormal condensation of heterochromatin but not of euchromatin. Previously, viable alleles had been identified which produced mutagen-sensitive and repair defective phenotypes (Boyd and Setlow, 1976; Brown and Boyd, 1981a,b). These findings suggested that the primary consequence of abnormal heterochromatin condensation leads to the secondary effects of mutagen sensitivity and repair defects (Gatti _et al_., 1983).

The possibility of an association between the apparently abnormal heterochromatin condensation and mutagen

sensitivity in some RS patients led to the investigation of mutagen sensitivity in RS+, RS-, RS+ obligate heterozygote and control cells (Gentner et al., 1985; 1986; Burns and Tomkins, 1989). Fibroblasts from RS+ patients were found to be hypersensitive to cell killing by a number of mutagenic agents, including mitomycin C, ultraviolet irradiation, cisplatin, 8-methoxypsoralen and methylnitrosourea(ibid.). In contrast, RS- and RS+ obligate heterozygote cells were found to be normally sensitive to mitomycin C (Burns and Tomkins, 1989). The mutagen sensitivity of RS+ cells has been most striking for crosslinking agents, although moderate sensitivity to acute gamma radiation and ethyl methanesulphonate (EMS) has been observed (Gentner et al., 1985; 1986; Van Den Burg and Francke, 1991; D.J. Tomkins, unpublished results).

The pedigree analyses of RS families have suggested that RS is inherited in an autosomal recessive manner (McKusick, 1990, 268300). The presence of the RS effect in some patients suggests that, at least in these patients, the abnormal gene also affects constitutive heterochromatin structure and function (Tomkins et al., 1979). In addition, the abnormal in vitro growth characteristics, mitotic abnormalities and mutagen hypersensitivity associated with the RS effect described in the previous sections may also be manifestations of this defect. The absence of all of these phenomena in cells from RS- patients suggests a difference

at the gene level between these two sub-groups of patients despite the lack of clinical differences. It has been found that in families where more than one sibling has been born with RS, the presence or absence of the RS effect is true-breeding (RS+: Mann et al., 1982; Robins et al., 1989; Maserati et al., 1991; RS-: Fryns et al., 1987). This observation is also suggestive of a genetic difference.

There are several genetic models involving autosomal recessive inheritance that could explain the apparent genetic heterogeneity in RS. The first model denies genetic heterogeneity and states that RS+ and RS- patients have the same mutation in the same gene. The true-breeding nature of the RS effect makes this the least likely of situations. Secondly, RS+ and RS- patients could result from allelic mutations in the same gene. This model of allelic heterogeneity suggests that the gene product responsible for RS has one domain that controls growth and limb development and other domain(s) responsible for mitotic function and heterochromatin structure and function. The RS- patients would have a point mutation in the first domain only whereas the RS+ patients would have a more severe mutation, perhaps a deletion, affecting the whole gene. One would hypothesize in this case that the RS- allele would retain some gene function and would thus be dominant to the RS+ allele. Finally, RS+ and RS- patients may arise from mutations in two different but related genes. This model of genetic

heterogeneity suggests that both genes affect the same biochemical pathway and thus result in the same clinical phenotype.

An alternative model is that of co-recessive inheritance (Lambert and Lambert, 1985, 1989, 1992). This model was proposed to explain the large number of complementation groups that have been identified in human DNA repair disorders such as xeroderma pigmentosum, Fanconi anaemia and ataxia telangiectasia. The model suggests that expression of certain diseases requires homozygosity or hemizygosity for two or more recessive alleles. It also predicts extremely high carrier frequencies for individual recessive alleles and that the requirement of two or more of these different recessive genes accounts for the rarity of these conditions. RS+ and RS- patients could be examples of co-recessive inheritance and share a defect in a gene that is common to both of their co-recessive inheritance systems. This common defect would account for the clinical similarities between RS+ and RS- patients while the other defective gene(s) would be responsible for the differences at the cellular level. This model could then be expanded to explain the clinical and cellular similarities between RS+ patients and Fanconi anaemia patients, a hypothesis that will be discussed more fully in the next section.

1.2 Fanconi Anaemia

In 1927, Dr. G. Fanconi described three brothers, all of whom were suffering from a severe familial anemia that was ultimately lethal. In addition, these siblings exhibited microcephaly, intensive brown pigmentation, hemorrhages of the skin, genital hypoplasia, internal strabismus and strongly increased reflexes. No decrease in mental capacity was noted (Fanconi, 1927). This condition was subsequently designated "Fanconi's Anaemia" (FA) (Naegeli, 1931). By 1967, more than 150 cases of Fanconi anaemia had been reported and Dr. Fanconi published a list of chief criteria of FA (Fanconi, 1967). The six most important criteria for a diagnosis of the disease were thought to be: 1) pancytopenia; 2) hyperpigmentation; 3) malformation, especially of the skeleton; 4) small stature since birth; 5) hypogonadism; and 6) familial occurrence. Criterion 6 was considered less critical if the patient also exhibited microcephaly, renal anomalies, hyperflexia, mental retardation and deafness (Fanconi, 1967). The International Fanconi Anaemia Registry (IFAR) was established in 1982 at The Rockefeller University to serve as a central repository for clinical, hematologic and genetic information on FA patients (Auerbach et al., 1989). The eight congenital malformations determined by the IFAR to be the most discriminatory for FA are growth retardation, birthmarks, kidney and urinary disorders, micropthalmia,

learning disabilities, low platelet counts, thumb and radial anomalies and other skeletal anomalies (Auerbach et al., 1989). In November 1992, it was reported that 370 patients have been enrolled in the American branch of the IFAR (Giampietro et al., 1992). A review of the skeletal anomalies observed in these patients reported that 26% exhibited abnormalities other than those of the thumb and radius, including abnormalities of the hips, vertebrae, ribs, feet, toes and metacarpals and phalangeals as well as scoliosis, spina bifida and craniosynostosis (ibid.). In addition, FA patients are at increased risk for developing malignancies, especially acute monocytic and myelomonocytic leukemia (Cervenka et al., 1981). It has been estimated that 15% of patients in the IFAR are leukemic or preleukemic, a percentage that is more than 15,000 times that in the general population (Auerbach, 1992).

The wide range of clinical variability in FA was noted by Dr. Fanconi in his 1967 review article. More recently, it has been observed that diagnosis solely on the basis of clinical manifestations is unreliable in FA and that cytogenetic analysis is required to confirm or reject the diagnosis of FA (Glanz and Fraser, 1982; Auerbach et al., 1989; Farrell et al., 1993). Indeed, 18.4% of FA patients in the American branch of the IFAR exhibit neither congenital malformations nor hematological disturbances and were ascertained solely on the basis of cytogenetic testing

performed because of an affected family member (Giampetro _et al_., 1992). Cytogenetic aspects of FA will be discussed in the next section.

Extensive family studies have concluded that FA is inherited in an autosomal recessive manner (Schroeder _et al_., 1976; McKusick, 1990, 227650). One study of approximately 90 FA families reported a non-significant difference in sex distribution of affected individuals, no maternal age effect, no clustering of affected siblings in birth order and no preference of higher birth orders (Schroeder _et al_., 1976). All of these had been mentioned as possibilities in previous reports (Fanconi, 1967). The presence of consanguinity in many affected families supports the theory of autosomal recessive inheritance in this rare condition (Fanconi, 1967). The carrier frequency for FA, which occurs equally in all ethnic groups, has been estimated to be approximately 1 in 200 (Auerbach, 1992). Interestingly, there have been reports of hematological symptoms or malformations in a number of relatives of FA patients including first degree relatives (parents, siblings and offspring) as well as more distant relatives (grandparents, aunts and uncles and others) (Gmyrek and Syllm-Rapoport, 1964 cited in Fanconi, 1967; Petridou and Barrett, 1990). These may represent either undiagnosed cases of FA, especially in siblings of affected individuals, or manifestations of the FA mutation in the heterozygous

state. Alternatively, the model of co-recessive inheritance could account for these cases in that these partially affected individuals may be exhibiting manifestations of homozygosity at one of the loci required for FA.

An increased level of spontaneous chromosome aberrations was first reported in lymphocyte cultures from two brothers affected with FA in 1964 and confirmed in later reports (Schroeder et al., 1964, Schroeder, 1966a,b; Schmid et al., 1965; Schmid, 1967). The vast majority of aberrations observed were chromatid-type aberrations such as gaps and breaks, chromatid exchange figures such as triradials and quadriradials and endoreduplication (Schroeder et al., 1964; Schmid, 1967). Chromosome-type aberrations occurred rarely in these cultures (ibid.). Subsequently, the presence of elevated spontaneous chromosome aberrations in suspected FA patients was considered diagnostic. It soon became apparent, however, that not all patients fitting the clinical picture of FA exhibited an increased level of spontaneous chromosome aberrations (Bushkell et al., 1976; Cervenka et al., 1981; Schroeder-Kurth et al., 1989; Auerbach et al., 1989). Thus, while the presence of elevated spontaneous chromosome aberrations in association with other predisposing factors (congenital malformations, hematological disturbances or affected family member) can be diagnostic of FA, its absence does not exclude the possibility that the patient is

afflicted with FA.

The classification of FA as a chromosomal breakage disorder led to speculation that there may be a defect in DNA repair associated with the elevated spontaneous chromosome aberrations observed in FA cells. Research into the sensitivity of FA lymphocytes to various mutagenic and carcinogenic agents revealed a specific hypersensitivity to bifunctional or polyfunctional crosslinking agents [tetrametansulfonil-d-mannit (R-52), mitomycin C (MMC), diepoxybutane (DEB), photoactivated psoralens], but not to other cytotoxic agents including monofunctional alkylating agents [decarbamoyl mitomycin C (DMCC), ethyl methanesulphonate (EMS)] and gamma irradiation (Schuler et al., 1969; Sasaki and Tonomura, 1973; Latt et al., 1975; Marx et al., 1983). This hypersensitivity was manifested by increased induced chromosome aberrations (ibid.). Exposure of FA fibroblasts to non-toxic levels of either DEB or EMS was also found to result in a significant increase in the level of chromosome aberrations (Auerbach and Wolman, 1976). Subsequently, it was reported that a significantly increased level of DEB-induced chromosome aberrations was also observed in fibroblasts from FA obligate heterozygotes (Auerbach and Wolman, 1978).

Clastogenic stress tests using DEB or MMC have become definitive tests for FA (Cervenka et al., 1981; Auerbach et al., 1981; Auerbach et al., 1985; Auerbach et

al., 1989). Although other diagnostic methods have been suggested for FA, the induction of chromatid-type chromosome aberrations by non-toxic doses of DEB is considered to be the most reliable and is the most widely used test for FA (Auerbach et al., 1989). Interestingly, despite early reports that FA heterozygotes could also be detected in this way, no consistent data could be obtained to support this claim (Cohen et al., 1982; Cervenka and Hirsch, 1983; Porfirio et al., 1983; Gebhart et al., 1985; Auerbach et al., 1989). Consequently, no cytogenetic test is currently available for heterozygote detection in FA.

The availability of a cytogenetic test that apparently unequivocally identified FA patients led to the realization that there was a proportion of patients who did not exhibit any congenital abnormalities but who suffered from FA (Schmid and Fanconi, 1978). The importance of differentiating these patients from patients with other forms of aplastic anaemia became evident when it was realized that the hypersensitivity of FA patients to certain chemotherapeutic agents was complicated by established cytoreduction regimens prior to bone marrow transplantation (Cervenka et al., 1981). That is, although bone marrow transplantation was a realistic alternative for patients with non-FA acute aplastic anaemia, the experience with FA patients was much less positive. Once the hypersensitivity of FA patients to certain agents such as cyclophosphamide,

which is regularly used in conditioning regimens, was established, however, regimens could be modified to take this hypersensitivity into account (Auerbach et al., 1983; Ebell et al., 1989; Gluckman et al., 1989).

Other manifestations of the hypersensitivity of FA cells to bifunctional DNA crosslinking agents have been reported. Cell killing assays with FA lymphocytes, fibroblasts and lymphoblastoid cells have revealed that these cells show an increased cellular sensitivity relative to control or obligate heterozygous cells to drugs such as MMC, photoactivated psoralen and DEB but not to other agents such as EMS (Finkelberg et al., 1974; Weksburg et al., 1979; Ishida and Buchwald, 1982; Wander and Fleischer-Reischmann, 1983; Poll et al., 1984; Auerbach et al.,1986; Duckworth-Rysiecki et al., 1985; Strathdee et al., 1992a). The degree of hypersensitivity in FA cells varied from several-fold to greater than 80-fold in some cases (ibid.).

The induction of sister chromatid exchanges (SCEs) by crosslinking agents has been an area of some controversy in FA. Initially, a hypoinduction of SCEs was reported in FA lymphocytes in response to treatment by MMC but not when treated with EMS (Latt et al., 1975; Cervenka et al., 1981). A corresponding hypoinduction was not observed in FA fibroblasts. Contrary to these findings, there have been many reports suggesting no difference in the induction rate of SCEs in FA cells compared to normal cells in response to

treatment with MMC, DEB, isoniazid, 4-nitroquinoline-1-oxide or nitrogen mustard (Novotna et al., 1979; Porfirio et al., 1983; Gebhart et al., 1985; Howell, 1991). Other studies have reported a hyperinduction of SCEs in FA lymphocytes by MMC or 4,5',8-trimethoxypsoralen (TMP) compared to control cells (Miura et al., 1983; Kano and Fujiwara, 1982). Thus, the issue of SCE induction in FA remains unresolved. However, given the genetic heterogeneity in FA, it is possible that the conflicting results obtained in these studies originate from genetic differences in the cells studied.

The wide range of clinical symptoms observed in FA patients has troubled clinicians for some time. Indeed, Fanconi commented that the large number, ubiquity and variability of clinical symptoms in FA were difficult to explain by a single gene defect (Fanconi, 1967). This clinical heterogeneity within Fanconi anaemia patients led to the hypothesis that genetic heterogeneity was a factor in this condition. The feasibility of using somatic cell hybridization as a method to detect complementation of the defects in FA cells was first established in fusions between normal fibroblasts and FA fibroblasts (Yoshida, 1980). Suppression of both the spontaneous and the MMC-induced chromosome aberrations in FA cells was found in hybrid cells thus indicating that these are recessive characteristics. Early somatic cell hybridization experiments using

fibroblasts from different FA patients confirmed that more than one complementation group was apparent within FA patients. That is, in some cases, hybrid cells exhibited correction of mitomycin C induced chromosome aberrations (Zakrzewski and Sperling, 1980). Interestingly, however, it was noted that in most cases complementation was not observed even from patients of diverse ethnic origins (Zakrzewski and Sperling, 1980, 1982; Zakrzewski et al., 1983; Yoshida, 1982). In addition, cells from patients with extreme clinical differences or whose cells exhibited different DNA repair characteristics failed to complement each other (Zakrzewski and Sperling, 1980, 1982; Zakrzewski et al., 1983). These observations led to the suggestion that clinical and cellular differences may be resulting from variable expressivity of a small number of mutations.

Subsequently, further complementation studies were performed which involved fusing lymphoblastoid cell lines from a number of FA patients with a universally hybridizing cell line from another FA patient. Patients were assigned to one of two complementation groups: those not capable of complementing the mitomycin C hypersensitivity in the universally hybridizing cell line or group A; and those capable of complementing group A cell lines or group B (also known as non-A) (Duckworth-Rysiecki et al., 1985). Recently, those cell lines previously assigned to group B were hybridized to each other and reassigned to one of three

complementation groups: B, C or D (Strathdee and Buchwald, 1991; Strathdee et al. 1992a). Thus, despite earlier findings, it is now known that at least four complementation groups are represented within the clinical condition known as Fanconi anaemia. It is not known whether differences in clinical presentation exist between the complementation groups. Differences with respect to mutagen sensitivity have not yet been established between the four complementation groups although differences between group A and those cell lines previously designated group B (non-A) have been reported (Moustacchi et al., 1987; Digweed et al., 1988).

Subsequent to the identification of the four complementation groups, attempts were made to clone the responsible genes using the novel approach of functional complementation. This approach has been successful in cloning the defective gene in group C patients, FACC (FA group C complementing) (Strathdee et al., 1992b). Functional complementation has also been used in attempts to isolate a cDNA capable of complementing the MMC and DEB sensitivities in FA group A fibroblasts (Moses et al., 1992). Candidate genes are now being examined for possible mutations.

Linkage studies using 34 FA pedigrees have provided some evidence of linkage to the long arm of chromosome 20 (Mann et al., 1991). This finding of linkage to 20q13 has subsequently been disputed by another group (Mathew et al.,

1991). However, given the genetic heterogeneity demonstrated in FA, it is likely that both groups have families representing different complementation groups included in their studies which would help to explain the equivocal results. It will be important to concentrate on patients in a single complementation group in future studies. Attempts to isolate a candidate gene for FA from 20q13 are currently underway (Allen et al., 1992).

Fanconi anaemia cells have been studied for the induction of mutations at two loci, the hypoxanthine-guanine phosphoribosyl transferase (HPRT) and the Na^{+}/K^{+} ATPase loci, by a number of different mutagens. FA fibroblasts from four different patients were shown to be hypomutable at these loci by both ethyl methanesulphonate (EMS) and mitomycin C (MMC) (Finkelberg et al., 1977). Recently, FA lymphoblastoid cells from complementation groups A and D (designated group B or non-A in publication) were shown to be hypomutable at both loci by photoactivated psoralens (Papadopoulo et al., 1990a,b). In addition, molecular analysis of $HPRT^{-}$ mutants from the FA group D cells revealed that while point mutations predominated in mutants from normal cells, the majority of mutations in the FA cells were small deletions (Papadopoulo et al., 1990b). Based on these observations, the authors postulated that FA group D cells are defective in an error-prone repair pathway that operates during replication via the bypass of unexcised mutagenic

lesions. Subsequently, it was shown that significantly fewer HPRT⁻ point mutants (as characterized by restriction enzyme digestion) from FA group D cells express mRNA. That is, 46 to 67% of spontaneous or psoralen-induced point mutants did not produce transcripts compared to 0 to 31% in normal cells (Guillouf _et_ _al_., 1991). Given the apparent predisposition of FA cells to deletion mutations, it was hypothesized that these mutants arose from the deletion of critical promoter sequences (_ibid_.; Papadopoulo _et_ _al_., 1990b). If the deletions were less than 100 bp in length, they would not be detectable using restriction enzyme digestion and thus appear to be point mutations. In any case, the hypomutability of FA lymphoblasts and the abnormal predominance of deletion and inactivating mutations in the mutants that are isolated are indicative of a difference in the process by which psoralen-induced lesions are repaired in FA cells compared to normal cells (Guillouf _et_ _al_., 1991).

It is now well established that FA cells possess some defect in the repair of certain kinds of DNA damage. The exact nature of this defect remains unknown. Cocultivation of normal human or FA cells from complementation groups A and D (designated B in publication) with either normal mouse lymphoma cells or "FA-like" mutant mouse lymphoma cells of two complementation groups, I and II, suggested that a diffusible factor released from normal

or FA group D human cells and from normal or group II mouse lymphoma cells was capable of correcting MMC-induced chromosome aberrations in group I mouse lymphoma cells and FA group A cells, respectively (Rosselli and Moustacchi, 1990). It is interesting that no reciprocal correction is seen on the FA group D cells or the group II mouse lymphoma cells thus suggesting a defect of a non-diffusible factor in these cell lines. When FA group A cells were cocultivated with group I mouse lymphoma cells, no correction was seen in either cell line, indicating analogous defects in these two cell lines (ibid.).

Heterologous DNA transfer experiments of mouse or Chinese hamster DNA into FA fibroblasts have provided some interesting insight into the origins of the mutagen-induced chromosomal instability and decreased clonogenic survival of FA cells (Diatloff-Zito et al., 1990; Shaham et al., 1987). Following mouse DNA-mediated gene transfer into FA fibroblasts from complementation group A, a near-complete correction of the MMC-induced chromosome aberrations was observed while no correction of the decreased clonogenic survival in response to MMC treatment was noted. Partial correction of both chromosomal and cellular sensitivity to 8-methoxypsoralen (8-MOP) plus UVA treatment was observed in these cells. In contrast, transfection into fibroblasts from complementation group D (designated group B in publication; same patient as HSC-62, Weksburg et al., 1979),

resulted in partial correction of the decreased clonogenic survival in response to MMC treatment but not in MMC-induced chromosome aberrations (Diatloff-Zito et al., 1990). The authors postulated that this dissociation of chromosomal instability and clonogenic survival suggested the presence of two domains in the protein(s) causing FA (ibid.; Diatloff-Zito et al., 1991; Moustacchi et al., 1991). Alternatively, the two cellular phenotypes could be caused by defects in two different, unlinked genes which, given that the transfectants incorporated only about 1% of the mouse genome, would not be co-transferred into the FA cells. Presumably, the cells would retain only that DNA necessary to survive selection in MMC which would be different for each of the two complementation groups. Partial correction of both chromosomal and cellular sensitivity to 8-MOP plus UVA treatment in transfected FA fibroblasts from complementation group A is probably a reflection of their much lower sensitivity to this mutagen (approximately 2-fold compared to 12- to 40-fold for MMC) and of the narrower spectrum of lesions induced (Weksburg et al., 1979; Ishida and Buchwald, 1982; Diatloff-Zito et al., 1986; Papadopoulo et al., 1987). Thus, correction of 8-MOP plus UVA-induced cellular and chromosomal damage may be more readily accomplished by a foreign wild-type gene than is MMC-induced damage (Diatloff-Zito et al., 1990).

In contrast, transfection of SV40 transformed FA

fibroblasts from a single patient with either normal human or Chinese hamster genomic DNA yielded cells resistant to DEB which exhibited correction of both the DEB-induced chromosome aberrations and decreased clonogenic survival in response to DEB treatment (Shaham et al., 1987). These results indicate that DNA from both humans and Chinese hamsters contain DNA sequences capable of correcting both of the abnormal cellular phenotypes in FA fibroblasts. Contrary to the findings with mouse DNA by Diatloff-Zito et al. (1990; 1991), these phenotypes do appear to be associated in both humans and Chinese hamsters. A second study investigating the ability of transfected genomic DNA from CHO, normal human fibroblast or FA fibroblast cells to restore normal MMC sensitivity to SV40 transformed FA fibroblasts yielded equivocal results because as many MMC-resistant colonies were obtained using FA DNA as the donor DNA as when normal or CHO DNA was used (Buchwald et al., 1987).

A large amount of work has been done in attempts to elucidate the exact nature of the DNA lesions that FA cells are incapable of correcting. A study of the cytogenetic toxicity of various antitumor platinum compounds in FA found that agents which introduced predominantly DNA-DNA crosslinks such as cis-platinum(II)-diamminedichloride or cis-platinum(IV)-diamminetetrachloride were much more cytotoxic to FA cells than was trans-platinum(IV)-

diamminetetrachloride, an agent which introduces predominantly DNA-protein crosslinks (Poll et al., 1982). Similarly, other studies have found that DNA-DNA crosslinks appear to be less efficiently repaired in FA than in control cells (Fujiwara et al., 1977; Fujiwara, 1982; Gruenert and Cleaver, 1985; Plooy et al., 1985; Averbeck et al., 1988; Matsumoto et al., 1989; Rousset et al., 1990; Sun and Moses, 1991). Because this deficiency has been observed both with agents inducing purine-purine (i.e. MMC) pyrimidine-pyrimidine crosslinks (i.e. 8-MOP), it is evident that an enzyme involved in the general removal of crosslinks is defective in FA rather than one specific for certain kinds of damage (Fujiwara et al., 1977; Fujiwara, 1982; Gruenert and Cleaver, 1985).

Observations that FA cells appear to be normally competent at repairing pyrimidine dimers and monoadducts, while in many cases being completely deficient in the removal of interstrand crosslinks, led to the suggestion that the defect in FA cells lies in the first half-excision step in the repair of interstrand crosslinks (Fujiwara et al., 1977; Fujiwara, 1982). This hypothesis was supported by the observation that FA fibroblasts are defective in the first, rapid component of SCE repair but not in the second slower component (these steps were found to correspond to the first half-excision and second monoadduct excision of crosslink repair, respectively) (Kano and Fujiwara, 1981).

Subsequent studies have shown a deficiency in FA cells in the repair of crosslinkable monoadducts (furan-side) induced by TMP plus 405 nm UVA treatment (Averbeck et al., 1988). However, reirradiation at 365 nm to increase the proportion of crosslinks compared to monoadducts increased the relative sensitivity of the FA cells compared to normals indicating that the repair of crosslinks was systematically hampered in FA cells (ibid.). The authors suggest that the DNA repair defect in FA cells is not expressed in response to minor distortions of the DNA double helix but is sensitive to major distortions such as those produced by TMP-induced monoadducts and crosslinks. That is, it is the degree of helical distortion produced by an adduct to which FA cells are hypersensitive rather than to the nature of the adduct itself (ibid.).

Differences in the repair of crosslinks between FA cells from different complementation groups have also been noted (Matsumoto et al., 1989; Rousset et al., 1990). Work with lymphoblastoid cell lines (LCLs) has shown that cells from FA complementation groups A, B and D (designated group B in publication) all exhibit increased sensitivity to MMC and a deficiency in the repair of DNA crosslinks. Within these three groups, however, it was found that an LCL from complementation group B was the most sensitive and a group D LCL was the least sensitive. Two group A LCLs which were comparable to each other in sensitivity exhibit an

intermediate response between group B and group D. Removal of crosslinks was found to correlate well with sensitivity data (Matsumoto et al., 1989). Similarly, a study of crosslink removal in fibroblasts from complementation groups A and D (designated group B in publication) revealed that two group A cell strains were significantly less efficient at crosslink repair than a group D strain which was slightly but not significantly less efficient than a control strain (Rousset et al., 1990). These data correlate well with those of the previous study and support the hypothesis of differences in crosslink repair in different FA cell strain/lines being the direct result of differences at the gene level. The efficiency of DNA excision repair in extracts from FA cell lines from complementation groups A and D (designated group B in publication) has also been studied (Hansson et al., 1991). These authors found no suggestion of an overall excision repair defect in cells from either group tested but could not exclude a defect in crosslink repair.

A number of enzymes involved in DNA replication and repair have been studied in FA cells as candidates for the basic defect in these cells. A difference in the intercellular distribution of DNA topoisomerase activity was noted in FA placental cells compared with cells from normal placentae (Wunder et al., 1981). In the case of the FA cells, the topoisomerase activity was concentrated in the

cytoplasmic subcellular fraction with very little activity in the nuclear fraction. The opposite was true in normal cells. The authors observed this discrepancy in three separate FA placentae and suggested that the defect in FA was not in the synthesis of the enzyme but rather in its transport across the nuclear membrane.

An altered nuclease has been reported in FA fibroblasts and lymphoblastoid cells from complementation group A (Sakaguchi et al., 1991; 1992). The mutant nuclease in the FA cells exhibits an isoelectric point (pI) of 6.8 compared to the normal pI of 6.3. The authors suggest that the defective gene in FA group A cells is responsible for the modification of the nuclease pI (Sakaguchi et al., 1991).

A study investigating chromatin-associated DNA endonuclease activity in lymphoblastoid cells from FA complementation groups A and D (designated group B in publication) revealed that these cell lines are defective in two different endonuclease activities (Lambert et al., 1992). In group A cells, an endonuclease with pI 4.6 exhibited 25% of the activity of the enzyme in normal cells while in group D cells, an endonuclease with pI 7.6 was found to be approximately 50% as active as the normal enzyme.

Finally, a decrease in the production of interleukin-6 (IL-6) has also been noted in FA fibroblasts

and lymphoblastoid cells from complementation groups A and D. The addition of IL-6 to culture medium was found to increase the FA cells resistance to MMC cytotoxicity (Rosselli et al., 1992). The authors suggested that the deficiency in IL-6 production could account for the hematopoietic defects in FA.

The observations by different researchers of different deficiencies in FA cells from the same complementation group suggest that the basic defect in FA cells is complex and may include many factors. The only FA gene to be cloned to date, FACC, has been found to encode a polypeptide that does not contain any motifs common to previously identified proteins and, thus, appears to represent a new protein involved in human DNA repair (Strathdee et al., 1992b).

1.3 Genetic Heterogeneity in Roberts Syndrome: Present Study

The clinical variability that has been recorded in the small number of reported cases of Roberts syndrome is extreme. The existence of RS patients with very few clinical manifestations compared to many patients who die in the perinatal period as a direct result of the many, severe manifestations they are afflicted with suggests that RS may be heterogeneous at the genetic level as well. In addition, the two cellular phenotypes exhibited in some patients, the

RS effect and mutagen hypersensitivity, are not universal to all RS patients. The present research project was designed to investigate heterogeneity in RS at several levels. It was hoped that complementation studies within RS using somatic cell hybrids would provide some information about the extent of genetic heterogeneity in RS. Complementation studies between RS and FA were used to explore the relationship between these two syndromes. In addition, mutability studies were designed to further investigate the similarity between RS and FA at the cellular level.

1.3.1 Genetic Heterogeneity in Roberts Syndrome

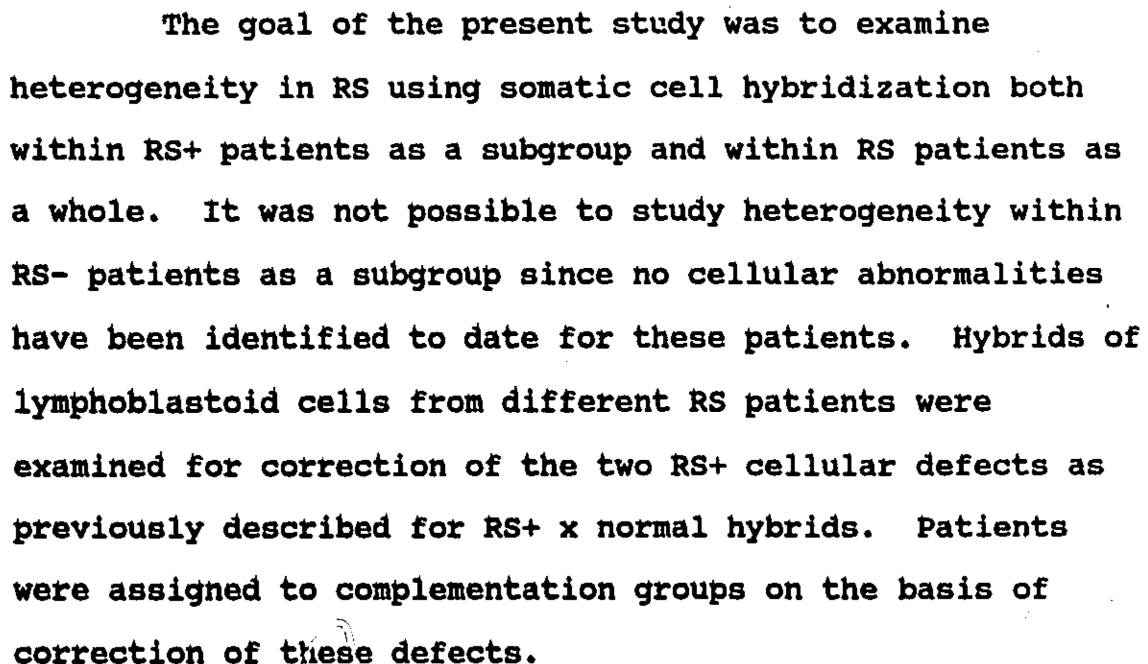
It has previously been demonstrated that the RS effect can be corrected by fusion of normal human or rodent cells with RS+ cells (Krassikoff et al., 1986; Knoll and Ray, 1986; Gunby et al., 1987). In addition, it has been demonstrated that those cells exhibiting the RS effect also exhibit a hypersensitivity to cell killing by mutagens such as MMC and cisplatin (Gentner et al., 1985; 1986; Burns and Tomkins, 1989). This association led to the hypothesis that the RS effect and the mutagen hypersensitivity may be manifestation of the same gene defect (Burns and Tomkins, 1989).

In the present study, somatic cell hybrids between RS+ and normal lymphoblastoid cells were examined for the RS effect and MMC sensitivity to determine if both of these

cellular phenotypes were corrected. Correction of both defects would strengthen the association between them and provide support for the hypothesis that they arise from the same mutation in the Roberts syndrome (RBS) gene.

The clinical heterogeneity in RS is suggestive of heterogeneity at the genetic level as well. The existence of two clinically indistinguishable groups that can be separated on the basis of cellular characteristics also suggests genetic heterogeneity. It is important to note, however, that the range of clinical variability is as great within RS- patients as it is within RS+ patients. Thus, genetic heterogeneity within each of these subgroups is also possible.

The goal of the present study was to examine heterogeneity in RS using somatic cell hybridization both within RS+ patients as a subgroup and within RS patients as a whole. It was not possible to study heterogeneity within RS- patients as a subgroup since no cellular abnormalities have been identified to date for these patients. Hybrids of lymphoblastoid cells from different RS patients were examined for correction of the two RS+ cellular defects as previously described for RS+ x normal hybrids. Patients were assigned to complementation groups on the basis of correction of these defects.



1.3.2 Relationship between Roberts syndrome and Fanconi anaemia

RS and FA patients share many characteristics both at the clinical level and at the cellular level. Both syndromes are characterized by growth retardation and by abnormalities of the limbs (Freeman *et al*., 1974; Auerbach *et al*., 1989). In both cases, radial anomalies are common (*ibid*.). While the variable mental retardation seen in RS has not been reported as often in FA, learning disabilities have been observed in some cases (Glanz and Fraser, 1982; Auerbach, 1992). Cardiac defects, renal anomalies and urogenital anomalies are seen occasionally in both syndromes (Glanz and Fraser, 1982; Auerbach, 1992; Waldenmaier *et al*., 1978; Mann *et al*., 1982; Feingold, 1992). Although hematologic disorders are not normally associated with RS, one case of thrombocytopenia has been reported (Waldenmaier *et al*., 1978).

At the cellular level, both FA patients and RS+ patients exhibit characteristic, albeit different, cytogenetic abnormalities. The increased level of chromosome aberrations in FA cells is not seen in RS+ (or RS-) patients nor is the RS effect evident in FA patients. On the other hand, cells from both FA patients and RS+ patients exhibit a cellular hypersensitivity that is specific for DNA crosslinking agents such as MMC and DEB. Consequently, both syndromes are considered to be possible

disorders of DNA repair.

FA fibroblast and lymphoblastoid cells have been shown to be hypomutable by ethyl methanesulphonate (EMS), MMC and photoactivated 8-methoxypsoralen (Finkelberg et al., 1977; Papadopoulo et al., 1990a,b). The mutagen hypersensitivity similarities between RS+ cells and FA cells led to the hypothesis that these cells may possess similar DNA repair defects and, thus, that RS+ cells may also exhibit hypomutability. The goal of the present study was to investigate RS+, RS-, FA and control lymphoblastoid cells for (i) survival in increasing dose of EMS as demonstrated by a limiting dilution cloning efficiency assay and (ii) mutability by increasing doses of EMS as measured by mutant frequency calculated from relative cloning efficiencies under selective and non-selective conditions. Mutant frequencies were compared at a standard point, the EC_{50} or the dose at which the relative cloning efficiency was 50%, for each cell line to eliminate the effect of differences in cell survival in comparisons.

The clinical and cellular similarities between FA and RS led to the speculation that these two conditions may be related at the DNA level. It has been suggested that they could be allelic syndromes (Hall, 1987). Alternatively, they could represent mutations in different but related genes which lead to a similar clinical manifestation. Finally, these two syndromes could represent

examples of co-recessive inheritance (Lambert and Lambert, 1985; 1989; 1992). That is, patients from the four complementation groups in FA and RS patients could share common recessive mutations in addition to other recessive mutations unique for each group or condition which explain the differences at both the clinical and cellular levels.

The present study investigated the genetic relationship between RS and FA using somatic cell hybrids between lymphoblastoid cells from an RS+ patient and patients representing each of the FA complementation groups identified to date. Hybrids were examined for correction of the RS effect, MMC hypersensitivity and hyperinduction of chromosome aberrations by DEB. Incomplete correction one or more of these abnormalities in hybrids may indicate a genetic association between RS and FA.

2. MATERIALS AND METHODS

2.1 Cell Lines

Lymphoblastoid cell lines (LCLs) from control, RS+, RS- and FA subjects were used in all aspects of this project. Control LCLs DM, JaKr and PeKr and all RS LCLs were derived in our laboratory by Epstein Barr virus transformation (Neitzel, 1986). The RS+ patients from whom R20 and LB-1 were derived have been described previously (Tomkins et al., 1979; Jurenka, 1976). Control LCLs HSC-3TO, HSC-55 and all FA LCLs were gifts from Dr. Manuel Buchwald, The Hospital for Sick Children, Toronto, Ontario. All of these LCLs have been described previously (Ishida and Buchwald, 1982; Buchwald et al., 1989). A cell line derived from a Lesch-Nyhan patient, GM1899A, was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Control cell line TK6 was a gift from Dr. Richard Albertini, University of Vermont, Burlington, Vermont and has been previously described (Levy et al., 1968; Skopek et al., 1978). A description of the LCLs used is given in Table 1.

Table 1: Lymphoblastoid Cell Lines Used in these Studies

Cell Line	Sex	Age of Donor (years)	Phenotype
HSC-3TO	M	Adult	Normal
DM	M	25	Normal
HSC-55	M	11	Normal
TK6	M	5	Normal
GM1899A	M	10	Lesch-Nyhan
JaKr	F	30	Fragile X carrier
PeKr	F	53	Fragile X carrier
LB-1	F	12	RS+
R20	M	7	RS+
TiBu	F	4	RS-
LoGi	F	12	RS-
HSC-72N	F	10	FA Group A
HSC-99	F	19	FA Group A
HSC-230N	M	3	FA Group B
HSC-536N	M	4	FA Group C
HSC-62N	M	2	FA Group D

2.2 Culturing of Lymphoblastoid Cell Lines

LCLs were maintained in RPMI-1640 medium (Gibco 380-2400AJ) supplemented with 15% or 10% fetal bovine serum (FBS; Gibco 200-6140AJ or Hyclone A-1115-L) for mutant (RS and FA) or control LCLs, respectively. Cultures were seeded at 2 to 5 x 10^5 cells per mL in T-25 (Falcon 3013) or T-75 (Corning 25110-75) flasks under maintenance conditions. Cells were maintained in a 37°C humidified incubator containing 5% CO_2. All LCL manipulation was performed in a Canadian Cabinets tissue culture hood with level B containment.

For long term storage, cells were frozen in liquid nitrogen. Cells were enumerated by trypan blue exclusion as follows: 20 µL of cell suspension was diluted in 180 µL of phosphate buffered saline (PBS; Gibco 310-4190AJ) (1:10 dilution); 20 µL of this suspension was diluted in 20 µL of trypan blue (Gibco 630-5250PE) (1:2 dilution; final dilution 1:20). A aliquot of this suspension was applied to either side of a hematocytometer (Reichert-Jung) and four squares on each side were counted at 400x magnification using a Leitz Laborlux 12 light microscope. Cell number per millilitre was calculated using the following equation:

$$\frac{\text{Total cell number}}{\text{8 squares}} \times 10^{4*} \times 20^{**} = \frac{\text{number of cells}}{\text{millilitre}}$$

* dilution factor per square of hematocytometer

** dilution factor per millilitre of cell suspension

Routinely, 1 x 10^7 viable cells were frozen in 1 mL of RPMI-

1640 supplemented with 15% FBS and 10% dimethyl sulphoxide (DMSO; BDH 2810) in a 1.5 mL screw-top cryovial (Diamed 222-3902-085).

To re-establish cultures from frozen aliquots, cells were thawed by agitating the vial by hand in a 37°C waterbath until partially thawed. One mL of prewarmed RPMI-1640 medium supplemented with 15% FBS was then added to the vial to completely thaw the suspension. This mixture was transferred to a 15 mL centrifuge tube containing a further 9 mL of medium. Cells were pelleted by centrifugation as previously described, all medium was aspirated and the pellet re-suspended in 1 mL of fresh medium. Cells were then counted as previously described and cultures were seeded at 2 - 5 x 10^5 cells per mL.

2.3 Introduction of pBabe- Selectable Markers

Two cell lines producing retroviral vector were used: PA317-neo which produces pBabe-neo particles and PA317-hygro which produces pBabe-hygro particles. These cell lines were gifts from Dr. Manuel Buchwald, The Hospital for Sick Children, Toronto, Ontario. The retroviral vectors, pBabe-neo and pBabe-hygro, contain genes conferring resistance to G418 and hygromycin B, respectively (Morgenstern and Land, 1990). These vectors have had the <u>env</u> sequences of the retrovirus deleted and, thus, require a packaging cell line to reproduce. PA317 is a "second

generation" packaging cell line derived from a murine fibroblast cell line with a packaging construct that lacks both a packaging sequence and a 3′ LTR (Morgenstern and Land, 1990). These deletions necessitate two recombination events between the packaging construct and the retroviral vector for wild type viruses to be produced, thus reducing, but not eliminating, the possibility of producing virus.

Prior to the collection of retroviral particles, it was necessary to subculture the PA317-neo or -hygro cells. The cells were grown as a monolayer in alpha minimal essential medium (α-MEM; Gibco 320-2571AJ) supplemented with 10% FBS in T-75 flasks. The cells were rinsed with PBS, subcultured by trypsinization (Gibco 610-5300AG) and reseeded at a 1:20 split ratio. When the monolayer was sub-confluent (1 to 2 days), the medium was replaced with 15 mL of fresh medium for 12 to 16 hours. The medium was harvested, centrifuged at 180 x g (1000 rpm) to remove any detached cells and the supernatant was distributed in 1 mL aliquots to 1.5 mL cryovials. The viral titre in this supernatant was approximately 10^6 colony forming units (CFU) per millilitre (Craig Strathdee, personal communication). Aliquots were frozen at -70°C until needed.

RS- LCLs were trans-infected with retroviral particles as follows: 1 mL of viral particles and 3 μg/mL polybrene (Sigma H9268) were added to 4 mL of RPMI-1640 medium supplemented with 15% FBS and containing

2×10^6 cells. For RS+ LCLs, 2 mL of viral particles and 3 µg/mL polybrene were added to 3 mL of medium containing 4×10^6 cells. Following a 48-hour incubation, the cells were transferred to a 15 mL tube and pelleted by centrifugation. The medium was aspirated and the pellet was re-suspended in 1 mL of fresh medium. A viable cell count was done as previously described and cultures were seeded at 5×10^5 cells/mL in fresh RPMI-1640 medium supplemented with 15% FBS. G418 (Sigma G5013) or hygromycin B (Sigma H8272) were added to a final concentration of 400 µg/mL. Cultures were incubated and reseeded to 5×10^5 cells/mL in fresh selection medium every 3 or 4 days. Because trans-infection efficiency is expected to be 20 to 50%, dead cells were diluted out of the culture as the resistant cells grew. When the culture reached a sufficient size, aliquots of 1×10^7 cells were frozen in liquid nitrogen as previously described. The trans-infections performed in this study are outlined in Table 2.

2.4 Complementation Analyses

2.4.1 Somatic cell hybridization of lymphoblastoid cells

A total of nine different pairs of parental LCLs were hybridized in seven different types of fusions. Table 3 outlines the parental pairs for each type of fusion and the number of fusion attempts for each pair. For each experiment, control fusions (i.e. parent to self) were also

Table 2: Trans-infection Experiments

Cell Line	Phenotype	Retroviral Vector	Resistant Cell Line
LB-1	RS+	pBabe-hygro	LB-1-*hygro*
R20	RS+	pBabe-neo	R20-*neo*
TiBu	RS-	pBabe-hygro	TiBu-*hygro*
LoGi	RS-	pBabe-hygro	LoGi-*hygro*

Table 3: Summary of Somatic Cell Hybridization Experiments

Parental Cell Lines Hybridized	Type of Hybridization	Number of Hybridization Attempts
HSC-3TO x LB-1	Control x RS+	4
HSC-3TO x R20	Control x RS+	2
R20-_neo_ x LB-1-_hygro_	RS+ x RS+	8
R20-_neo_ x LoGi-_hygro_	RS+ x RS-	4
R20-_neo_ x TiBu-_hygro_	RS+ x RS-	2
LB-1-_hygro_ x HSC-72N	RS+ x FA A	2
LB-1-_hygro_ x HSC-230N	RS+ x FA B	4
LB-1-_hygro_ x HSC-536N	RS+ x FA C	2
LB-1-_hygro_ x HSC-62N	RS+ x FA D	2

performed for each of the parental LCLs.

Hybridization conditions were identical for all experiments. Five million cells from each of the parental LCLs were mixed in a 50 mL centrifuge tube. Fresh RPMI-1640 medium supplemented with 15% FBS was added to a final volume of 10 mL. Cells were sedimented by centrifugation at 50 x g (600 rpm) for 5 minutes. All medium was aspirated and the pellet was re-suspended in 10 mL of serum-free minimal essential medium (Gibco 410-1800EB). Cells were again sedimented by centrifugation as previously described and the medium aspirated. The tube was placed in a beaker of water at 37°C and 1 mL of 45% w/v polyethylene glycol (PEG-1000, Baker U-218-7 for control x RS+ experiments; PEG-1450, Sigma P7777 for all other experiments) was added to the pellet over 60 seconds. The tube was incubated at 37°C for a further 60 seconds. Two millilitres of serum-free medium was added over two minutes followed by an additional 8 mL over 2 to 3 minutes. Cells were sedimented by centrifugation as previously described and all PEG-containing medium aspirated. The pellet was resuspended in 10 mL of fresh RPMI-1640 medium supplemented with 15% FBS and 1 mL of this suspension was distributed into each of 10 wells of a 24-well tissue culture plate (Nunclon or Costar 3524). The plate was incubated for 24 hours.

Following the 24-hour incubation, 0.5 mL of 3x selection medium was added to each well of the plate. For

the control x RS+ fusions, the selection medium was comprised of 10^{-6} mol/L ouabain (Sigma O3125) and 2% HAT supplement (Sigma H0262; 5×10^{-3} mol/L hypoxanthine, 2×10^{-5} mol/L aminopterin, 8×10^{-4} mol/L thymidine). This medium selected for the ouabain resistance and against the 6-thioguanine resistance of the HSC-3TO cells. The RS+ cells are resistant to HAT but sensitive to ouabain. Thus, only hybrid cells can survive in this double selection medium. For all other fusions, the selection medium was comprised of 400 μg/mL of G418 (Sigma G5013) and 400 μg/mL hygromycin B (Sigma H8272). Those LCLs containing the neomycin-resistance gene were resistant to G418 but sensitive to hygromycin B while those containing the hygromycin B-resistance gene were resistant to hygromycin B but sensitive to G418. Thus, again, only hybrid cells can survive in this double selection medium. In all cases, medium was half-changed every 2 to 3 days with fresh 1x selection medium until a change in medium colour was observed to indicate hybrid growth (2 to 4 weeks). Hybrid cell lines (HCLs) were expanded for use in cytogenetic and mitomycin C (MMC) sensitivity analyses.

2.4.2 Confirmation of hybrids by VNTR analysis

Genomic DNA was extracted from 5×10^6 to 1×10^7 live cells of each of the parental LCLs and the HCLs for variable number tandem repeat (VNTR) studies. Cells were

washed once with PBS in a 1.5 mL microcentrifuge tube (Diamed PRE150-R) and the pellet was resuspended in 500 μL of 1x lysis buffer containing 100 mmol/L sodium chloride (NaCl; BDH), 50 mmol/L Tris (pH 7.5; Boehringer Mannheim 604-205), 1 mmol/L EDTA (pH 8.0; Sigma E5134) and 0.5% sodium dodecyl sulphate (SDS; Biorad 161-0301). Tubes were incubated at 37°C until the pellet was completely dissolved. Proteinase K (BRL 5530UA) was added to a final concentation of 1 μg/μL (50 μL of 10 mg/mL stock solution) and tubes were incubated at 37°C overnight or at 55°C for 3 hours. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) (phenol: Gibco 5513UA; chloroform: Caledon 3000-1; isoamyl alcohol: Fisher A-393) once with 24:1 chloroform:isoamyl alcohol. DNA was precipitated in a 1.5 mL screw-cap tube (Biorad 224-0110) by the addition of 200 mmol/L sodium acetate (NaAc; BDH) and 2.5 volumes of ice-cold (-20°C) 100% ethanol (EtOH). The pellets were washed several times with ice-cold 100% EtOH, air-dried, dissolved in 50 to 300 μL of Tris-EDTA (TE; pH 8.0) and stored at 4°C.

In preparation for VNTR analysis, approximately 5 μg of genomic DNA was digested overnight at 37°C with the restriction enzyme _Hae_III (Boehringer Mannheim 693936).

All VNTR analyses were done by Dr. John Waye, Department of Pathology, McMaster University, using established protocols (Nakamura _et al_., 1987; Budowle and

Baechtel, 1990). For the RS+ x control hybridizations, the pattern of fragment sizes recognized by the probe D10S28 (Bragg et al., 1988) was compared between the parental LCLs and the HCLs. For the remaining hybridizations, the probes D4S139 and D17S79 were used (Milner et al., 1989; Balazs et al., 1989).

2.4.3 Cytogenetic analysis of hybrids

Cytogenetic analysis for the RS effect was carried out in all parental LCLs and HCLs. The induction of chromosome aberrations by diepoxybutane (DEB) was investigated in at least two HCLs from each of the RS+ x FA hybridizations. For both, cells were subcultured to a density of 2×10^5 cells/mL in a 10 mL culture of RPMI-1640 supplemented with 15% FBS in a T-25 flask 48 hours prior to the desired time of harvesting. For the induction of chromosome aberrations DEB (Sigma D7019) was added to a final concentration of 0.1 μg/mL at the time of culture initiation and the cultures were protected from light in a foil wrapped metal basket. For both cytogenetic analyses, colcemid (Gibco 120-5212AD) was added to a final concentration of 100 ng/mL for the last three hours before harvesting.

Cultures were then transferred to a 15 mL centrifuge tube and sedimented by centrifugation. Medium was aspirated to 0.5 to 1 mL and the pellet resuspended in the remaining

supernatant. Cells were swollen for 12 minutes in pre-warmed 0.07 mol/L potassium chloride (BDH) at 37°C and again sedimented by centrifugation and resuspended as previously described. The cells were fixed by the addition of freshly prepared ice-cold 3:1 methanol:glacial acetic acid fixative to a final volume of 6 mL for at least 30 minutes at 4°C. The cells were sedimented by centrifugation as previously described and the pellet washed twice with fresh ice-cold fixative.

Slides were made by dropping 1 to 2 drops of cell suspension onto ice-cold methanol-washed double-frosted glass slides. The slides were blown to distribute the cell suspension evenly over the slide, flamed briefly over a methanol burner and allowed to dry on a hot plate. Slides were coded although the presence of the RS effect made it impossible to score cytogenetic abnormalities in a completely blinded fashion. Slides were stained in a 5% solution of Giemsa stain (Gurr-BDH 35086) made up in 10% Gurr buffer pH 6.8 (BDH 33193) for 3 to 5 minutes, rinsed well with distilled, deionized water and allowed to air-dry.

All parental LCLs and HCLs were assessed for the presence or absence of the RS effect. Twenty-five cells from each cell line were scored at 1000x magnification. For each cell, its location, chromosome number and the presence or absence of the RS effect were noted.

Chromosome aberrations were scored in each of fifty

metaphase spreads. For each cell, location, chromosome number, and the number of each of the following aberrations observed were noted: chromosome gap, chromosome break, dicentric, ring chromosome, chromatid gap, chromatid break, triradial figure, quadriradial figure, complex re-arrangement, de-spiralization and pulverization.

All photographs were taken using a Perceptive Scientific Instruments (PSI) automated karyotype machine.

2.4.4 Mitomycin C dose response analysis

All parental LCLs and all HCLs were assessed for MMC sensitivity by chronic exposure. In addition, HSC-3TO was included in all experiments as an internal control. Cells were seeded into each well of a 24-well plate at 1 to 2.5 x 10^5 cells/0.5 mL. For the control x RS+ hybridizations, 24 replicate wells were seeded. For all other hybridizations, 28 replicate wells were seeded. MMC (Sigma M0503) was added at 2x the desired final concentration in 0.5 mL RPMI-1640 supplemented with 15% FCS. The final concentrations of MMC were 0, 10, 30, 100, 300, 1000 and 3000 ng/mL for HSC-3TO, the RS+ parental LCLs and all HCLs except for R20-neo x LB-1-hygro HCL 2 (3000 ng/mL dose was not used in control x RS+ experiments). This cell line plus the FA parental LCLs from complementation groups A, B and D were treated with doses of 0, 3, 10, 30, 100, 300 and 1000 ng/mL. The FA group C parental cell line,

HSC-536N, was the most sensitive and was treated with doses of 0, 1, 3, 10, 30, 100 and 300 ng/mL. Four replicate wells of each dose were treated. The plates were incubated for 6 to 9 days or until growth was evident in the 0 dose wells by a change in medium colour.

At the end of the incubation period, the cells were transferred from the wells into 5 mL, plastic counting tubes (Sarstedt 55-476-005) using a siliconized pasteur pipette. Cultures were mixed well, diluted 1:2 with trypan blue and viable cells were enumerated as previously described.

This determination was performed for all four replicates of each dose for each cell line. The relative cell number for each well was determined by taking the number of cells/millilitre for that well and dividing it by the number of cells/millilitre in the corresponding 0 dose replicate. Thus, the cell number of the 0 dose replicate was considered to be 100% and the relative cell number for the first replicate of the 10 ng/mL dose was calculated by dividing its cell number by the cell number for the first replicate of the 0 ng/mL dose, the second replicate is divided by the second replicate of the 0 dose and so on. Replicates were defined by their position in the 24-well plate. That is, the first row of wells constituted the first replicate, the second row constituted the second replicate, and so on.

2.5 Mutability of Roberts Syndrome Cells by Ethyl Methanesulphonate

Table 4 outlines the LCLs used in these experiments and the assays performed for each cell line. In cases where the LCL was already resistant to 6-thioguanine (6-TG), only its limiting dilution cloning efficiency after ethyl methanesulphonate (EMS) exposure was determined.

In each experiment, two LCLs were exposed to five increasing doses of EMS: 0, 100, 150, 200 and 250 μg/mL. It has been estimated that to ensure that statistically adequate numbers of 6-TG-resistant mutant cells survive treatment when the treatment has no mutagenic effect, at least 80 mL of cell culture at a density of 5 x 10^5 cells/mL must be treated (Furth et al., 1981). For each LCL, cultures were expanded until at least 2 x 10^8 cells were obtained. The cultures were subcultured to 5 x 10^5 cells/mL in 80 mL cultures two days prior to the desired treatment time to ensure that the cultures were in log growth at the time of treatment. The cultures were then transferred to 50 mL centrifuge tubes and sedimented by centrifugation at 180 x g (1000 rpm) for 10 minutes. All medium was aspirated and each pellet was resuspended in 5 to 10 mL of fresh RPMI-1640 medium supplemented with 15% FBS and 1% penicillin-streptomycin (Gibco 600-5070AG). All suspensions for each LCL were pooled and a small aliquot was taken to count. Cell numbers were enumerated as previously described and

Table 4: Lymphoblastoid Cell Lines Used in EMS Studies

Cell Line	Phenotype	Sex	Cloning Efficiency	Mutability
DM	control	M	yes	yes
HSC-55	control	M	yes	yes
HSC-3TO	control	M	yes	no
GM1899A	control	M	yes	no
JaKr	control	F	yes	yes
PeKr	control	F	yes	yes
LB-1	RS+	F	yes	yes
R20	RS+	M	yes	yes
TiBu	RS-	F	yes	yes
LoGi	RS-	F	yes	yes
HSC-72OT	FA A	F	yes	no
HSC-99	FA A	F	yes	yes

five 79 mL cultures were seeded with 4×10^7 cells/culture. EMS (Sigma M0880) was diluted to 80x the desired dose in medium and 1 mL of the appropriate dose was added to each culture. The cultures were incubated for 24 hours.

Following the 24 hour incubation period, cells were sedimented by centrifugation as previously described and the pellets for each dose of each LCL were resuspended in 5 to 10 mL of fresh RPMI-1640 medium supplemented with 15% FBS and 1% penicillin-streptomycin. Cells were enumerated and a 200 µL aliquot taken for cloning efficiency determination. The remaining culture was reseeded in fresh medium at 5×10^5 cells/mL to a maximum of 80 mL per culture. The cells in the aliquot set aside for the cloning efficiency determination were diluted to 2 to 64 cells/100 µL, the cell number determined by limiting dilution assays as the optimal cell number per well for each LCL to give sufficient positive wells for statistical accuracy (Lefkovits and Waldmann, 1984; Waldmann and Lefkovits, 1984). This cell suspension was then distributed to each well of two-96 well microtitre plates (Costar 3799) per dose per LCL (100 µL/well). Each of these plates already contained 100 µL of fresh medium or 100 µL of medium containing 1×10^4 gamma irradiated (1×10^4 rads) TK6 cells which acted as a feeder layer. In some experiments, four microtitre plates were seeded per dose, two with a feeder layer and two without. The plates were incubated for 2 to

4 weeks or until control (untreated) plates showed sufficiently large colonies for scoring.

Prior to the selection of mutants deficient for hypoxanthine-guanine phosphoribosyl transferase (HPRT), it was necessary to culture the cells under non-selective conditions to allow for their recovery from the cytotoxic effects of EMS and for the expression of mutations at the HPRT locus. It has been reported that expression time for such mutations in human cells is approximately 6 days (Furth et al., 1981), thus, this was the minimum period that cells were cultured under non-selective conditions. During this recovery/expression phase, cultures were maintained in log growth by subculturing to 5 x 10^5 cells/mL in a 80 mL culture every two days. Treated cultures were considered to have recovered from the cytotoxic effects of EMS when their growth was similar to that of the untreated dose. This period was usually 7 to 9 days in control or RS- LCLs lines and 14 to 21 days in RS+ LCLs.

At the end of the recovery/expression period, cells were sedimented by centrifugation at 180 x g (1000 rpm) for 10 minutes, and the pellets for each dose of each LCL were resuspended in 5 to 10 mL of medium. Cell numbers for each culture were determined as previously described. A 200 μL aliquot from each culture was taken and diluted to 2 to 64 cells/100 μL and was distributed to 96 well microtitre plates as previously described for non-selection cloning

efficiency determination. The remaining cell suspension was diluted to 4 x 10^4 cells/100 μL for cloning efficiency determination under selective conditions. 6-Thioguanine (Sigma A4882) was added to a final concentration of 10 μg/mL (6 x 10^{-5} mol/L). One hundred microlitres was distributed to each well of as many 96 well microtitre plates as could be seeded depending on the cell number. Again, each microtitre well already contained 100 μL of either plain medium or 100 μL of medium containing 1 x 10^4 irradiated TK6 feeder cells. Thus, the final concentration of 6-TG in each well was 5 μg/mL (3 x 10^{-5} mol/L). In some experiments, selection plates were split so that one-half of the plates contained feeder cells and one-half did not. All microtitre plates, non-selection and selection, were incubated for 2 to 4 weeks or until sufficient colony growth was observed in the non-selection plates of the untreated culture.

At the end of the incubation period, each well of each plate was scored for colony growth using a Bausch and Lomb Photozoom inverted microscope at 40x magnification. The number of positive wells per plate were noted at two separate scoring timepoints several days apart. The number obtained in the second scoring was used in all calculations as it was felt that wells that were ambiguous at the first scoring could be scored more definitively as positive or negative at the second scoring thus giving more accurate results.

Cloning efficiency was calculated using an equation for Poisson distributed data:

$$A = \frac{\ln [T/(T - P)]}{B}$$

where A is the cloning efficiency, T is the total number of wells scored, P is the total number of positive wells and B is the number of cells seeded per well.

The cloning efficiency for each dose determined immediately post-exposure to EMS treatment was expressed relative to that of the untreated culture. Thus, the cloning efficiency of the untreated culture was considered to be 100% and the relative cloning efficiency of each of the treated cultures was determined as follows:

$$\text{relative cloning efficiency} = \frac{\text{A (treated culture)}}{\text{A (untreated culture)}}$$

Mutant frequency was calculated from the data obtained using the cloning efficiencies under non-selective and selective conditions following the recovery/expression period. Mutant frequency was calculated as follows:

$$\text{mutant frequency} = \frac{\text{selection cloning efficiency}}{\text{non-selection cloning efficiency}}$$

2.6 Statistical Methods

For all statistical analyses, a level of significance (α) of 0.05 was chosen.

2.6.1 Statistical analysis of cytogenetic results

The number of cells that were scored as RS+ for each

LCL was compared to those of the control LCL, the appropriate parental LCLs and the appropriate HCLs for each of the fusions performed using chi-square analysis. In cases where the expected frequencies were very low, Yates' correction for continuity was used (Ferguson, 1966).

Two different analyses were done on the chromosome aberration data using Minitab 7.2. First, the number of cells with and without chromosome aberrations were compared between LCLs and HCLs using chi-square analysis. Secondly, the mean number of aberrations per cell was compared between LCLs and HCLs using two-sample Student's t-tests.

2.6.2 Regression analysis of MMC dose response and EMS cloning efficiency data

Regression analysis was used to fit equations to the MMC dose response data and the EMS relative cloning efficiency data. Minitab 7.2 (Minitab Reference Manual, Release 7, 1989) was used to perform these analyses. For the MMC dose response data, the relative cell number data were $\log_{10}$ transformed and plotted against the $\log_{10}$ transformed MMC doses. These transformations were performed for each of the four replicates for each LCL. Regression analysis was used to fit an equation to the plot of the data. It was found that for each LCL, the data best fit the equation:

$\log_{10}$ relative cell number =

$$c + b \times \log_{10} \text{ mitomycin C dose}$$

where b is the slope and c is the intercept (constant). For each replicate, an equation was fit to the data and from this equation, the D_{10} value (the value at which 10% survival is estimated) for each replicate was calculated.

For the relative cloning efficiency data, the untransformed relative cloning efficiency was plotted against EMS dose and an equation was fitted to this plot. It was found that the data best fit the equation:

$$\text{relative cloning efficiency} = c + b \times \text{EMS dose}$$

where b is the slope and c is the intercept (constant). For each experiment, an equation was fit to the data for each LCL and the EC_{50} (the dose at which 50% relative cloning efficiency is estimated) was calculated.

2.6.3 Statistical analysis of mitomycin C D_{10} values

The MMC D_{10} values for all replicates of each LCL in each set of experiments (those experiments in which the same LCLs were tested) were compared between LCLs using Tukey's wholly significant difference (WSD) test (Miller, 1981). It was necessary to use a multiple comparisons test because the responses of four to six LCLs were being compared simultaneously. In these analyses, the effects of cell line, replicate number and experiment number on the D_{10} values were determined using Statistix 3.5 (Analytical

Software, 1991). For each cell line, the dose reduction factor (DRF) of the estimated D_{10} value of each replicate was calculated as follows:

$$DRF = \frac{D_{10}\ HSC\text{-}3TO}{D_{10}\ cell\ line}$$

A mean DRF with standard deviation was calculated for each cell line. In each set of experiments, the mean DRF for each class of cell lines (i.e. control, RS+, RS-, FA or hybrid) was compared to a standard value of 1.00 using a Student's t-test with Minitab 7.2.

2.6.4 Statistical analysis of ethyl methanesulphonate EC_{50} values using Student's t test

The mean EMS EC_{50} values for the RS+ LCLs, the RS- LCLs and the FA group A LCLs were compared to the mean EC_{50} value for the each of the control LCLs by calculating the dose reduction factor (DRF) as follows:

$$DRF = \frac{mean\ EC_{50}\ control\ LCL}{mean\ EC_{50}\ LCL}$$

The mean DRF for each LCL and each class of LCLs (RS+, RS- and FA) was compared to a standard value of 1.00 using a Student's t-test. These analyses were performed using Minitab 7.2.

2.6.5 Statistical analysis of ethyl methanesulphonate EC_{50} mutant frequency data

Given the large amount of variability in the mutant frequency between replicates, it was necessary to fit a model

to the EMS mutant frequency data using a regression method that would be resistant to extreme outliers in the data set. Accordingly, these data were fit to a model using a robust regression estimation method known as the Welsch regression estimate using Minitab 7.2 (Staudte and Sheather, 1990). Repeated iterations of Welsch bounded influence regression estimates were calculated using ordinary least squares as starting values. Each of the dependent values was weighted and a coefficient for each of the independent variables was calculated in each iteration. The iterations were repeated until the difference between the last two sets of Welsch estimates was zero to two decimal places. The dependent values were the mutant frequency values divided by 10^{-6} (to remove the exponential function). The main independent variables used in these calculations were the EMS dose in micrograms per millilitre and the EMS dose squared. In addition, columns were used as indicators of experiment number. That is, a separate column was created for each experiment and a value of one was entered beside each mutant frequency value for that experiment while a zero was entered beside each value that was not from that experiment. Thus, an equation is given which has a coefficient for dose, a coefficient for $dose^2$ and a coefficient for each experiment:

$$\text{mutant frequency}/10^{-6} = d + c \times \text{dose} + b \times \text{dose}^2 + a_1 \times \exp 1 + \ldots. a_n \times \exp n$$

where c is the coefficient for dose, b is the coefficient for

$dose^2$, a_1 to a_n are the coefficients for experiment 1 to experiment n (n is the number of replicate experiments) and d is the intercept (constant). This model was chosen because some of the cell lines produced a quadratic-like function when mutant frequency was plotted against dose. All of the cell lines fit this model quite well. Data from experiments when a feeder layer was not used were analysed separately from data when a feeder layer was used.

The EC_{50} mutant frequency for each experiment was calculated by taking the EC_{50} value for that experiment and entering it into the equation for the cell line, using only the appropriate terms for that experiment. An EC_{50} mutant frequency was estimated for each replicate experiment and a mean EC_{50} mutant frequency was determined for all experiments when a feeder layer was not used and for all experiments in which a feeder layer was used.

For the RS-, RS+ and FA LCLs, a mutant frequency reduction factor (MFRF) was calculated for each of the two mean EC_{50} mutant frequencies (i.e. with or without a feeder layer) relative to each of the same sex control cell lines. That is, because a difference of at least one order of magnitude was noted between the mutant frequencies of cell lines of opposite sex, it was felt that the most accurate comparison would be between cell lines of the same sex. Thus, for R20, MFRFs were calculated relative to the EC_{50} mutant frequencies of each of the two male control LCLs, DM and HSC-

55 while for the remaining LCLs, MFRFs were calculated relative to the EC_{50} mutant frequencies of the female control LCLs, JaKr and PeKr. The MFRF was calculated as follows:

$$MFRF = \frac{EC_{50} \text{ mutant frequency (control)}}{EC_{50} \text{ mutant frequency (LCL)}}$$

The mean MFRFs for each of the RS-, RS+ and FA LCLs and for each class of LCLs were compared to a standard value of 1.00 using a Student's t-test.

3. RESULTS

3.1 Genetic Heterogeneity in Roberts Syndrome

3.1.1 RS+ x control studies

The purpose of these somatic cell hybridization experiments was to determine if both the RS effect and mitomycin C (MMC) hypersensitivity cellular phenotypes of RS+ cells could be corrected by fusion with normal cells. The results of the RS+ x control fusion experiments are presented in Table 5. Four replicate hybridizations of fusion 1 (LB-1 x HSC-3TO) were performed and two replicate hybridizations of fusion 2 (R20 x HSC-3TO) were performed. A total of 36 hybrid cell lines (HCLs) were obtained for fusion 1 and 20 HCLs were obtained for fusion 2. No HCLs were obtained for any of the control fusions. Three HCLs from three different replicates of fusion 1 were chosen for mitomycin C (MMC) and cytogenetic analyses while one HCL from each of the two replicates of fusion 2 were tested. All five of these HCLs were shown by variable number tandem repeat (VNTR) analysis using the probe, D10S28, to be true hybrids of the fused parental LCLs (data not shown).

The results of the cytogenetic analyses of the three parental LCLs, HSC-3TO, LB-1 and R20 and the 5 HCLs are

Table 5: Results of the RS+ x Control Hybridization Experiments

Fusion Number	Parental Cell Lines	Number of Replicates	Number of HCLs Isolated	Number of HCLs Analyzed
Fusion 1	HSC-3TO x LB-1	4	36	3
Fusion 2	HSC-3TO x R20	2	20	2

presented in Table 6. The parental LCLs were all found to be hyperdiploid while the HCLs were hypertetraploid. As indicated in Table 6, 100% of the cells scored for the two RS+ LCLs were found to exhibit the RS effect. In contrast, none of the cells scored for either the control LCL or the five HCLs exhibited the abnormality. Chi-square analyses demonstrated that the proportion of cells exhibiting the RS effect in the RS+ LCLs was highly significantly greater than that of the control LCL or those of any of the HCLs ($p < 0.0001$). In addition, when the data for the two RS+ LCLs were pooled and compared to that of the control LCL, the pooled data for the LB1x3TO HCLs or the pooled data for the R20x3TO LCLs, the proportion of affected cells was again highly significantly greater for the RS+ LCLs ($p < 0.0001$). The correction of the RS effect in the HCLs is demonstrated in Figure 1. The normal, tightly apposed appearance of the paracentromeric regions and nucleolar organizing regions of HSC-3TO chromosomes is shown in Figure 1A while the abnormal appearance of these regions in RS+ cells is indicated in Figure 1B. As demonstrated in Figure 1C, heterochromatin structure appears normal in the HCL.

The mean D_{10} values estimated from fitted regression equations for the mitomycin C dose responses of each cell line are presented in Table 7. In addition, the dose reduction factor (DRF) for each cell line and for each group (class) of cell lines is given. Of the three factors

Table 6: Results of the Cytogenetic Analyses for RS+ x Control Hybridization Experiments

Cell Line	Modal Chromosome Number (min-max)	Number of Cells Scored	Cells with RS Effect (%)
HSC-3TO	47 (45 - 49)	25	0
LB-1	51 (47 - 54)	25	100
R20	49 (45 - 50)	25	100
LB1x3TO HCL 1	96 (92 - 99)	50	0
LB1x3TO HCL 2	96 (91 - 98)	50	0
LB1x3TO HCL 3	96 (91 - 102)	50	0
R20x3TO HCL 1	93 (91 - 96)	50	0
R20x3TO HCL 2	96 (90 - 99)	50	0

Figure 1: The appearance of constitutive heterochromatic regions in parental LCLs and HCLs from fusion 2. Chromosome 1 and an acrocentric chromosome are indicated by arrows in each photograph. A. Metaphase spread from the control LCL, HSC-3TO. B. Metaphase spread from R20. The Y chromosome is indicated by the arrowhead.

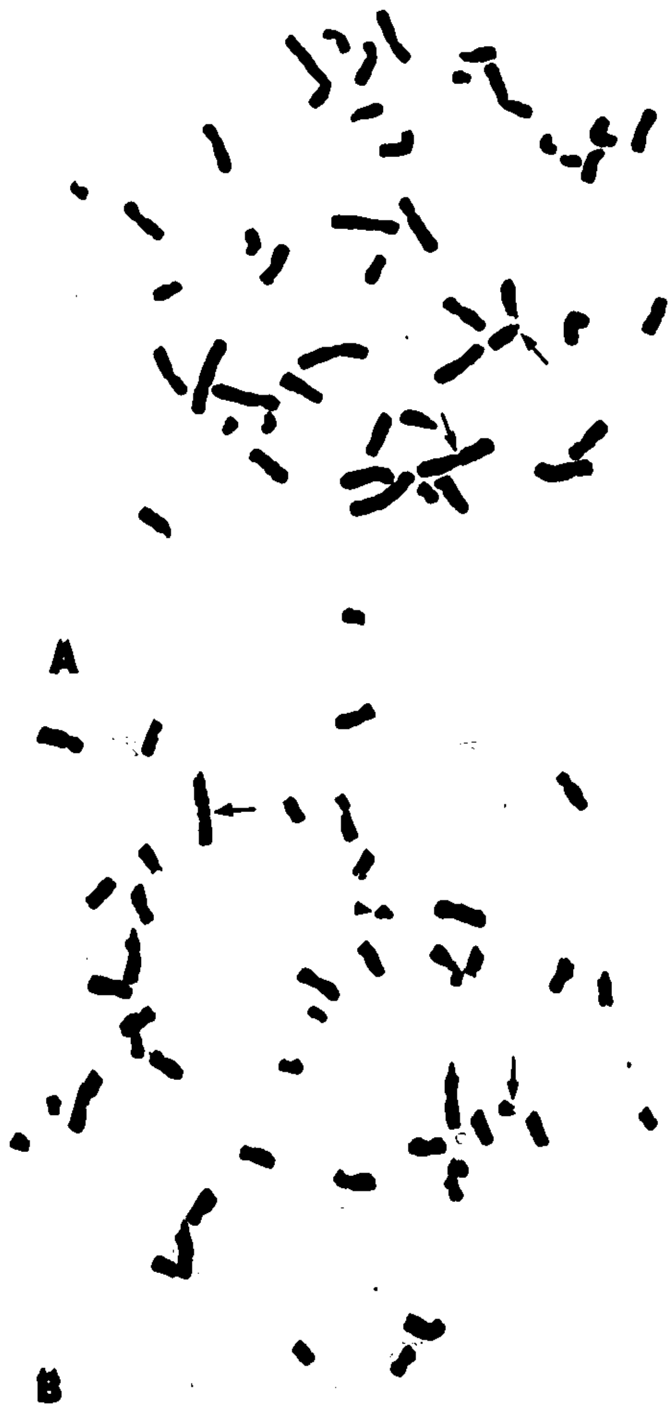

A
B

Figure 1 (cont'd): The appearance of constitutive heterochromatic regions in parental LCLs and HCLs from fusion 2. Chromosome 1 and an acrocentric chromosome are indicated by arrows in each photograph. C. Metaphase spread from R20x3TO HCL 2.

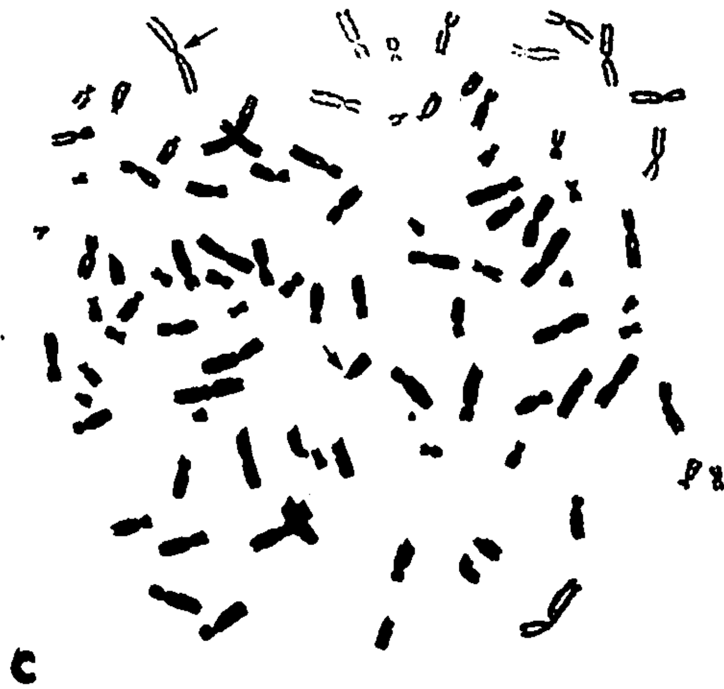

c

Table 7: Mean Fitted Mitomycin C D_{10} Values and Dose Reduction Factors for RS+ x Control Hybridization Experiments

Cell Line	Mean D_{10}* (sd) (ng/mL)	Mean DRF** (sd)	Mean DRF for class (sd)	P value for mean = 1.00
HSC-3TO	115.00 (33.90)	1.00	1.00	1.0000
LB-1	42.61 (13.10)	2.91 (1.08)	2.81 (1.12)	<0.0001
R20	45.73 (13.20)	2.72 (1.22)		
LB1x3TO HCL 1	168.03 (43.30)	0.74 (0.30)	0.98 (0.68)	0.8816
LB1x3TO HCL 2	103.71 (27.43)	1.16 (0.41)		
LB1x3TO HCL 3	149.80 (98.48)	1.16 (0.87)		
R20x3TO HCL 1	280.77 (63.00)	0.44 (0.22)		
R20x3TO HCL 2	95.71 (30.24)	1.42 (0.91)		

* D_{10} = the mitomycin C dose at which 10% survival is estimated to occur from the fitted regression equation

** Dose Reduction Factor (DRF) = $\frac{D_{10}\ \text{HSC-3TO}}{D_{10}\ \text{cell line}}$

tested in the analysis of variance, only cell line was found to have a significant effect ($p < 0.0001$) while the effects of experiment number and replicate number were not found to be significant ($p = 0.0563$ and $p = 0.7240$, respectively). The results of the Tukey's wholly significant difference (WSD) test were somewhat confusing. It was found that, at $\alpha = 0.05$, LB1x3TO HCL 2 was significantly less sensitive to MMC than were any of the other HCLs or the parental LCLs. In addition, LB1x3TO HCL 1, LB1x3TO HCL 3 and both R20x3TO HCLs were found to have mean D_{10} values not significantly different from that of HSC-3TO. However, the mean D_{10} values of HSC-3TO, R20x3TO HCL 2 and LB1x3TO HCL 3 were also found to be not significantly different from those of the two RS+ LCLs, R20 and LB-1. Given that the mean D_{10} values of both RS+ were more than two-fold lower than that of any of these three cell lines, it was felt that the low power of this test may be causing a type II statistical error (the failure to detect a difference when one is present). Unfortunately, the power function for the studentized range statistic, the measure used in the Tukey's WSD comparison, is unknown (Miller, 1981) so the power of the comparisons could not be established. Accordingly, a two-sample Student's t-test was performed between the mean D_{10} values of these five cell lines. It was found that while the mean D_{10} values for R20 and LB-1 were not significantly different from each other ($p = 0.6392$), they were significantly lower

than those of HSC-3TO ($p \leq 0.0004$), R20x3TO HCL 2 ($p \leq 0.0003$) and LB1x3TO HCL 3 ($p \leq 0.0021$). The mean D_{10} values for HSC-3TO and the two HCLs were not significantly different from each other ($p \geq 0.2516$). The mean DRF for the RS+ lines (2.81) was found to be significantly higher than 1.00 (the DRF for HSC-3TO) ($p < 0.0001$) while the mean DRF for the HCLs (0.98) was not significantly different from 1.00 ($p = 0.8816$).

In summary, the results of the cytogenetic and MMC sensitivity analyses of the RS+ x control HCLs suggest that they are completely corrected for both the RS effect and MMC hypersensitivity of RS+ cells.

3.1.2 RS+ x RS+ study

The purpose of this somatic cell hybridization experiment was to determine if RS+ patients from two diverse populations belong to the same complementation group. The results of the RS+ x RS+ fusion experiments are summarized in Table 8. Fusion 3 (R20-<u>neo</u> x LB-1-<u>hygro</u>) was attempted eight times. No HCLs were obtained in seven of the eight attempts but two HCLs were obtained in the other attempt. Both of these HCLs were analysed for the RS effect and MMC sensitivity. Hybridization attempts were performed in pairs with one control fusion (parent to self) for each of the parental LCLs in each experiment. Thus, a total of four control fusions were performed for each parental LCL. No

Table 8: Results of the RS+ x RS+ and RS+x RS- Hybridization Experiments

Fusion Number	Parental Cell Lines	Number of Replicates	Number of HCLs Isolated	Number of HCLs Analyzed
Fusion 3	R20-neo x LB-1-hygro	8	2	2
Fusion 4	R20-neo x TiBu-hygro	2	4	3
Fusion 5	R20-neo x LoGi-hygro	4	2	2

HCLs were obtained in any of these control fusions. The R20xLB1 HCLs studied were both shown by VNTR analysis using the probe D4139 to be true hybrids (data not shown).

Table 9 outlines the results of the cytogenetic analyses of the parental LCLs and the R20xLB1 HCLs with respect to the RS effect. All parental LCLs were observed to be hyperdiploid and both HCLs were hypertetraploid. It was found that 96 to 100% of the cells analysed for the two RS+ LCLs exhibited the RS effect. Similarly, 100% of the cells analysed for the R20xLB1 HCLs were found the exhibit the abnormality. None of the cells analysed for HSC-3TO were found to show the RS effect. Chi-square analyses demonstrated a highly significantly greater proportion of cells exhibiting the RS effect in the two RS+ LCLs and in the two R20xLB1 HCLs than in the control LCL ($p<0.0001$). The apparent lack of correction in the R20xLB1 HCLs is demonstrated in Figure 2. This cell from one of the HCLs displays the puffing of the constitutive heterochromatin that is characteristic of the RS effect.

The mean calculated mitomycin C D_{10} values for each of the cell lines are presented in Table 10. The mean DRFs for each cell line and for each class of cell lines are also given. The effect of cell line on the D_{10} value was found to be highly significant ($p < 0.0001$) while neither experiment number nor replicate number were found to exert a significant effect ($p = 0.1084$ and $p = 0.6009$,

Table 9: Results of Cytogenetic Analyses for the RS Effect in RS+ x RS+ and RS+ x RS- Hybridization Studies

Cell Line	Modal Chromosome Number (min- max)	Number of Cells Scored	Cells with RS Effect (%)
HSC-3TO	47 (45 - 49)	25	0
TiBu-*hygro*	46 (45 - 47)	25	0
LoGi-*hygro*	46 (45 - 47)	25	0
R20-*neo*	48 (45 - 51)	25	96
LB1-*hygro*	52 (46 - 54)	25	100
R20xLB1 HCL 1	104 (96 - 106)	25	100
R20xLB1 HCL 2	97 (95 - 101)	25	100
R20xTiBu HCL 1	93 (90 - 97)	25	0
R20xTiBu HCL 2	94 (92 - 97)	25	4
R20xTiBu HCL 3	96 (93 - 103)	25	0
R20xLoGi HCL 1	93 (89 - 99)	25	0
R20xLoGi HCL 2	95 (84 - 98)	25	0

Figure 2: The appearance of the constitutive heterochromatic regions in R20xLB1 HCL 1. Affected regions of chromosome 1, a D group chromosome and a G group chromosome are indicated by arrows.

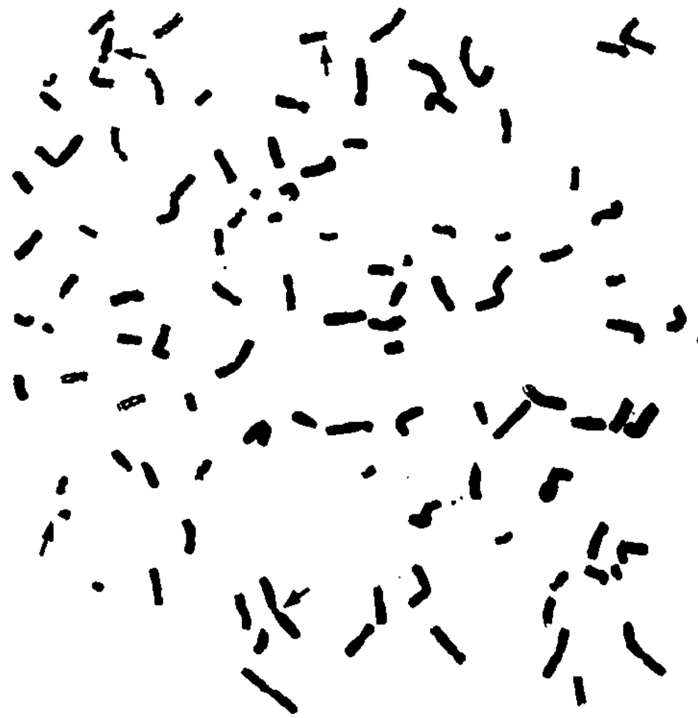


Table 10: Mean Fitted Mitomycin C D_{10} Values and Dose Reduction Factors for RS+ x RS+ Hybridization Experiments

Cell Line	Mean D_{10}* (sd) (ng/mL)	Mean DRF** (sd)	Mean DRF for class (sd)	P value for mean = 1.00
HSC-3TO	99.65 (27.80)	1.00	1.00	1.0000
LB-1-<u>hygro</u>	42.09 (17.86)	2.84 (1.53)	3.14 (1.42)	<0.0001
R20-<u>neo</u>	30.05 (4.74)	3.44 (1.28)		
R20xLB1 HCL 1	34.09 (15.88)	4.11 (1.28)	4.47 (1.68)	<0.0001
R20xLB1 HCL 2	17.97 (4.95)	4.84 (1.32)		

* D_{10} = the mitomycin C dose at which 10% survival is estimated to occur from fitted regression equation

** Dose Reduction Factor (DRF) = $\frac{D_{10}\ \text{HSC-3TO}}{D_{10}\ \text{cell line}}$

respectively). The mean D_{10} value for HSC-3TO was found to be significantly higher than those of the RS+ LCLs and the R20xLB1 HCLs ($\alpha = 0.05$). The mean D_{10} value for R20xLB1 HCL 1 was not significantly different from that of either of the RS+ LCLs. In contrast, R20xLB1 HCL 2 was found to have a D_{10} value significantly lower than that of LB-1-hygro but not significantly different from those of R20-neo or R20xLB1 HCL 1. The mean DRF for the RS+ LCLs (3.14) was found to be significantly greater than 1.00 ($p < 0.0001$). Similarly, the mean DRF for the R20xLB1 HCLs (4.47) was significantly higher than 1.00 ($p < 0.0001$). These results suggest that LB-1 and R20 are incapable of correcting the defects in each other and therefore belong to the same complementation group.

3.1.3 RS+ x RS- Studies

The purpose of these somatic cell hybridization experiments was to determine if RS- were capable of complementing the RS effect and MMC hypersensitivity in RS+ cells. The results of the RS+ x RS- fusion experiments are given in Table 8. For fusion 4 (R20-neo x TiBu-hygro), two replicate hybridizations were performed and for fusion 5 (R20-neo x LoGi-hygro), four replicate hybridizations were attempted. One HCL was obtained for the first replicate of fusion 4 and 3 HCLs were isolated from the second replicate. Two of the four attempts for fusion 5 failed to yield any

HCLs but 1 HCL was obtained from each of the other two replicates. Three control fusions were performed for R20-neo, one control fusion was performed for TiBu-hygro and two control fusions were performed for LoGi-hygro. No HCLs were isolated from any of the control fusions. The single HCL from the first replicate of fusion 4 and two HCLs from the second replicate were chosen for cytogenetic and mitomycin C sensitivity analyses. Both of the HCLs of fusion 5 were analysed. All of the HCLs studied were shown by VNTR analysis with the probe D4139 to be true hybrids (data not shown).

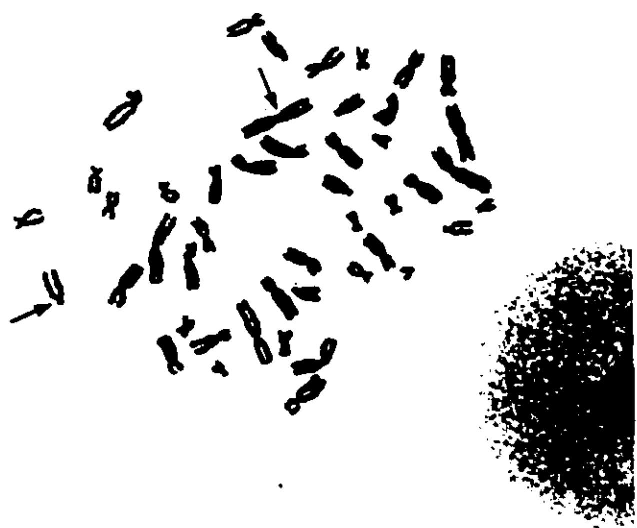
The results of the cytogenetic analyses for the RS+ x RS- fusions are given in Table 9. All parental LCLs were found to be hyperdiploid and all HCLs were hypertetraploid. For the RS+ LCL, R20-neo, 96% of the cells examined exhibited the RS effect while none of the cells analysed for the RS- LCLs exhibited it. No cells exhibiting the RS effect were observed in 4 of the 5 HCLs studied. The other HCL, R20xTiBu HCL 2 was observed to exhibit abnormal heterochromatin appearance in one group G chromosome of one cell of the 25 cells examined. Chi-square analyses demonstrated that the RS+ LCL exhibited the RS effect in a highly significantly greater proportion of its cells than did the control LCL, the two RS- LCLs or any of the HCLs ($p < 0.0001$). The appearance of the constitutive heterochromatin in the RS- cells and the RS+ x RS- HCLs is

shown in Figure 3. Figure 3A shows a cell from one of the RS- LCLs, TiBu-*hygro*, and Figure 3B shows an RS+ x RS- hybrid cell. In both cases, the appearance of the constitutive heterochromatin is normal.

The mean estimated mitomycin C D_{10} value for each of the cell lines is given in Table 11. In addition, the mean DRF for each cell line and for each class of cell lines is given. For fusion 4, the effect of cell line on the D_{10} values was highly significant ($p < 0.0001$) while neither experiment number nor replicate number were found to have a significant effect ($p = 0.7367$ and $p = 0.5003$, respectively). The mean D_{10} value of the RS- LCL, TiBu-*hygro*, was not significantly different from that of HSC-3TO. In addition, the mean D_{10} values for R20xTiBu HCL 1 and R20xTiBu HCL 2 were found to be not significantly different from those of HSC-3TO and TiBu-*hygro*. The mean D_{10} value for R20xTiBu HCL 3 was not significantly different from those of the other HCLs nor was it significantly different from that of HSC-3TO. However, this HCL's D_{10} value was significantly higher than that of TiBu-*hygro*. The mean D_{10} value for the RS+ LCL, R20-*neo*, was significantly lower than those for all of the other cell lines ($\alpha = 0.05$).

For fusion 5, all three factors tested were found to have a significant effect on the D_{10} values. The effects of cell line and experiment number were both highly significant ($p < 0.0001$) while the effect of replicate number was

Figure 3: The appearance of the constitutive heterochromatic regions of cell lines from fusion 5. Chromosome 1 and a D group chromosome are indicated by arrows in each photograph. A. Metaphase spread from TiBu-*hygro*; B. Metaphase spread from R20xTiBu HCL 2.



A

B

Table 11: Mean Fitted Mitomycin C D_{10} Values and Dose Reduction Factors for RS+ x RS- Hybridization Experiments

Cell Line	Mean D_{10}* (sd) (ng/mL)	Mean DRF** (sd)	Mean DRF for class (sd)	P value for mean = 1.00
HSC-3TO	102.29 (28.25)	1.00	1.00	1.0000
R20-neo	52.44 (12.13)	2.03 (0.60)	2.03 (0.60)	<0.0001
TiBu-hygro	98.21 (13.56)	1.18 (0.20)	1.21 (0.37)	0.0204
LoGi-hygro	102.68 (38.61)	1.23 (0.46)		
R20xTiBu HCL 1	120.50 (25.35)	1.00 (0.31)	0.87 (0.28)	0.0040
R20xTiBu HCL 2	116.89 (31.63)	1.05 (0.32)		
R20xTiBu HCL 3	148.11 (34.10)	0.79 (0.12)		
R20xLoGi HCL 1	118.83 (33.76)	0.64 (0.18)		
R20xLoGi HCL 2	152.61 (62.10)	0.86 (0.26)		

* D_{10} = the mitomycin C dose at which 10% survival is estimated to occur from fitted regression equation

** Dose Reduction Factor (DRF) = $\frac{D_{10}\ \text{HSC-3TO}}{D_{10}\ \text{cell line}}$

significant ($p = 0.0380$). The mean D_{10} values for HSC-3TO and the RS- LCL, LoGi-hygro, were not significantly different from each other nor were they significantly different from that of R20xLoGi HCL 1. In addition, the mean D_{10} values for the two HCLs tested were not significantly different from each other. The mean D_{10} value for R20xLoGi HCL 2, however, was significantly higher than those for HSC-3TO and LoGi-hygro. The mean D_{10} value for the RS+ LCL, R20-neo was significantly lower than those of all of the other cell lines ($\alpha = 0.05$).

The mean DRF for R20-neo (2.03) was highly significantly different from 1.00 ($p < 0.0001$). The mean DRF for the RS- LCLs (1.21) was also found to be significantly higher than 1.00 ($p = 0.0204$). However, a two-sample Student's t-test comparing the mean DRFs of the RS+ and RS- LCLs showed that the DRF for R20-neo was significantly higher than that of the RS- LCLs ($p < 0.0001$). The mean DRF for the RS+ x RS- HCLs (0.86) was significantly lower than 1.00 ($p = 0.0040$) thus indicating that these cell lines are less sensitive than the control cell line.

The results of these analyses suggest that the normal heterochromatin structure and MMC sensitivity in RS- cells are dominant to the defects in RS+ cells.

3.2 Similarities Between Roberts Syndrome and Fanconi Anaemia

3.2.1 Cloning efficiency immediately post-exposure to ethyl methanesulphonate

The purpose of these experiments was to determine the relative ethyl methanesulphonate (EMS) sensitivities of control, RS+, RS- and FA LCLs as measured by their limiting dilution cloning efficiency. The mean EMS EC_{50} values for the cell lines tested in the cloning efficiency experiments are presented in Table 12. The most notable difference observed among the control cell lines is the low EC_{50} value exhibited by DM compared to those of the other controls. Indeed, DM was 2- to 3-fold more sensitive to EMS treatment than were any of the other controls (DRF = 2.61, $p = 0.0016$). The mean DRF for the RS- LCLs was 0.89, a value that is not significantly different from 1.00 ($p = 0.2554$). Similarly, when the mean DRF for each of the RS- LCLs was calculated separately (TiBu = 1.01; LoGi = 0.78), neither was significantly different from 1.00 ($p = 0.9696$ and $p = 0.0944$ for TiBu and LoGi, respectively). Thus, the RS- LCLs were not found to be significantly more or less sensitive to EMS treatment than was the control group. The mean DRF for the RS+ LCLs was 2.00 which was found to be highly significantly different from 1.00 ($p = 0.0005$). When the mean DRF for each RS+ LCL was

Table 12: Fitted EMS EC_{50} and DRF Values for Control, RS-, RS+ and Fanconi Anaemia Cell Lines

Cell Class	Cell Line	Number of Replicates	Mean EC_{50}* (Line) (sd)	Mean EC_{50} (Class) (sd)	Mean DRF** (Class) (sd)	P value for DRF = 1.00
Control	DM	8	74.3 (29.3)	173.8 (58.0)	1.00	n/a
	HSC-55	1	167.3			
	JaKr	3	236.2 (42.5)			
	PeKr	1	149.4			
	HSC-3TO	1	202.4			
	GM1899A	1	213.1			
RS-	TiBu	1	172.9	197.8 (35.1)	0.89 (0.30)	0.2554
	LoGi	2	222.6 (72.8)			
RS+	R20	3	101.2 (10.8)	88.6 (17.8)	2.00 (0.71)	0.0005
	LB-1	5	76.0 (9.2)			
FA Group A	HSC-72OT	1	93.2	106.5 (18.7)	1.66 (0.57)	0.0022
	HSC-99	1	119.7			

* EC_{50} = the dose at which 50% relative cloning efficiency is estimated to occur from fitted regression equation

** DRF = dose reduction factor = $\frac{EC_{50} \text{ control LCL}}{EC_{50} \text{ LCL}}$

calculated separately (R20 = 1.72; LB-1 = 2.29). both were significantly different from 1.00 (p = 0.0298 and p = 0.0091 for R20 and LB-1, respectively). Thus, both RS+ LCLs are significantly more sensitive to EMS treatment than is the control group. The mean DRF for the two FA LCLs was 1.66 which is significantly different from 1.00 (p = 0.0022). When the mean DRF was calculated separately for each of the FA LCLs, that of HSC-72OT (1.87) was significantly different from 1.00 (p = 0.0190) while that of HSC-99 (1.45) was not (p = 0.0706). Thus, while the FA LCLs taken as a group were found to be significantly more sensitive to EMS treatment than was the control group, only HSC-72OT was significantly more sensitive than the control group when they were tested separately although with a sample size of two, the power to detect a difference may not have been adequate.

These results suggest that RS+ LCLs are significantly more sensitive to EMS treatment than are control or RS- LCLs. FA LCLs from complementation group A also show an increased sensitivity.

3.2.2 Mutability of Roberts syndrome cells by ethyl methanesulphonate

The purpose of these experiments was to determine the relative mutability of control, RS+, RS- and FA complementation group A LCLs by EMS. A summary of the mean mutant frequency values in the absence of a feeder layer at

the mean EC_{50} value calculated from fitted regression equations for each LCL tested is presented in Table 13. In addition, the mean EC_{50} mutant frequency value for each class of LCLs is given. Finally, the mutant frequency reduction factor (MFRF) at the mean EC_{50} compared to sex-matched control LCLs is given for each class of LCLs.

The mean MFRF for the RS- LCLs, LoGi and TiBu, was 0.66, a value that was significantly lower than 1.00 ($p = 0.0422$) When the mean MFRF was calculated separately for LoGi and TiBu it was 0.50 and 0.83, respectively. For LoGi, this value was significantly lower than 1.00 while for TiBu, it was not significantly different from 1.00 ($p = 0.0412$ and $p = 0.1952$, respectively).

For the female RS+ LCL, LB-1, the mean MFRF was 7.04, a value that was significantly greater than 1.00 ($p = 0.0489$). The male RS+ LCL, R20, failed to yield any mutants at any of the doses used in three replicate experiments. The EC_{50} mutant frequency for this LCL, therefore, was $<9.90 \times 10^{-5}$ (based on <1 mutant per dose) and its minimum mean MFRF was estimated to be 13.54. However, due to the large standard deviation of the mean MFRF (15.25), it was not significantly different from 1.00 ($p = 0.4530$). It must be noted that the actual MFRF may be much larger than this estimated minimum. The estimated minimum mean MFRF for the RS+ LCLs as a class was 10.29 which was not significantly different from 1.00 ($p = 0.1478$)

Table 13: Mutant Frequencies at the EMS EC_{50} Values for Control, RS-, RS+ and Fanconi Anaemia Cell Lines in the Absence of a Feeder Layer

Cell Line	Mean EC_{50}* (sd)**	Mean $MF(EC_{50})$*** (line) (sd)	Mean $MF(EC_{50})$ (class) (sd)	Mean MFRF**** (class) (sd)	P Value for Mean MFRF = 1.00
DM	74.33 (29.34)	2.75×10^{-4} (1.35×10^{-4})	1.35×10^{-3} (1.52×10^{-3})	1.00	1.0000
HSC-55	167.60	2.42×10^{-3}			
JaKr	236.20 (42.48)	5.34×10^{-5} (3.78×10^{-5})	5.72×10^{-5} (5.30×10^{-6})	1.00	1.0000
PeKr	149.40	6.09×10^{-5}			
LoGi	222.60 (72.83)	1.15×10^{-4} (2.12×10^{-5})	9.20×10^{-5} (3.25×10^{-5})	0.66 (0.20)	0.0422
TiBu	172.90	6.90×10^{-5}			
LB-1	75.99 (9.21)	8.12×10^{-6} (7.30×10^{-6})	$<5.38 \times 10^{-5}$ (6.46×10^{-5})	>10.29 (9.58)	0.1478
R20	101.20 (10.8)	$<9.95 \times 10^{-5}$§ (4.55×10^{-5})			
HSC-99	119.71	4.70×10^{-5}	4.70×10^{-5}	1.22 (0.11)	0.2250

* EC_{50} = the dose at which 50% relative cloning efficiency is observed
** s.d. = standard deviation
*** $MF(EC_{50})$ = the mutant frequency at the EC_{50} value
**** MFRF = mutant frequency reduction factor = $\frac{MF(EC_{50})\ \text{control LCL}}{MF(EC_{50})\ \text{LCL}}$

§ maximum $MF(EC_{50})$ based on <1 mutant per experiment

due to its large standard deviation (9.58). The lowest estimated MFRF for this class was 2.76 while the greatest was 24.32. Both of these estimates were for R20.

The MFRF for the LCL from FA complementation group A, HSC-99, was 1.22 and was not significantly different from 1.00 ($p = 0.2300$).

The results of the mutability experiments in the presence of an irradiated TK6 feeder layer are presented in Table 14. Once again, the mean mutant frequency at the EC_{50} value is given for each LCL as well as for each class of LCLs. In addition, the mean MFRF of each class of LCLs compared to sex-matched controls is given.

In the presence of a feeder layer, the mean MFRF for the RS- LCLs, LoGi and TiBu, was 1.08 and was not significantly different from 1.00 ($p = 0.9266$). The individual mean MFRF were 0.02 and 2.15 for LoGi and TiBu, respectively. Thus, LoGi was again found to be significantly hypermutable compared to its controls ($p = 0.0093$) while the mutability of TiBu was not significantly different from that of the controls ($p = 0.5666$).

The mean MFRF for the RS+ LCLs, LB-1 and R20, in the presence of a feeder layer was 28.47 but was not significantly different from 1.00 ($p = 0.3516$) due to an extremely large standard deviation (49.89). The individual mean MFRF were 5.28 and 51.67 for LB-1 and R20,

Table 14: Mutant Frequencies at the EMS EC_{50} Values for Control, RS-, RS+ and Fanconi Anaemia Cell Lines in the Presence of a Feeder Layer

Cell Line	Mean EC_{50}* (sd)**	Mean MF(EC_{50})*** (line) (sd)	Mean MF(EC_{50}) (class) (sd)	Mean MFRF**** (class) (sd)	P Value for Mean MFRF = 1.00
DM	159.80	3.05×10^{-4}	6.67×10^{-2} (9.38×10^{-2})	1.00	1.0000
HSC-55	755.60	1.33×10^{-1}			
JaKr	286.43 (183.32)	2.14×10^{-4} (2.33×10^{-4})	1.29×10^{-4} (1.20×10^{-4})	1.00	1.0000
PeKr	286.55 (129.90)	4.45×10^{-5} (6.29×10^{-5})			
LoGi	339.90	5.90×10^{-3}	2.98×10^{-3} (4.13×10^{-3})	1.08 (1.68)	0.9266
TiBu	133.10	6.02×10^{-5}			
LB-1	63.89 (4.30)	2.45×10^{-5} (2.53×10^{-5})	6.57×10^{-4} (8.95×10^{-4})	28.47 (49.89)	0.3516
R20	109.93 (16.44)	1.29×10^{-3} (1.84×10^{-4})			
HSC-99	104.20	6.44×10^{-5}	6.44×10^{-5}	2.01 (1.86)	0.5822

* EC_{50} = the dose at which 50% relative cloning efficiency is observed
** s.d. = standard deviation
*** MF(EC_{50}) = the mutant frequency at the EC_{50} value;
**** MFRF = mutant frequency reduction factor = $\frac{MF(EC_{50})\ control\ LCL}{MF(EC_{50})\ LCL}$

respectively. However, the very large standard deviations for the MFRFs (4.89 and 72.74, respectively) resulted in them being not significantly different from 1.00 ($p = 0.4320$ and $p = 0.5032$, respectively).

The FA group A LCL, HSC-99, was found to have a MFRF of 2.01 in the presence of a feeder layer. This value was not significantly different from 1.00 ($p = 0.5822$).

The raw data for these studies are presented in Appendix II. For each LCL, the absolute cloning efficiency immediately after EMS treatments, the absolute non-selection cloning efficiency following the expression/recovery period, the absolute selection cloning efficiency and the mutant frequency are given for each experiment. The data for experiments in the absence or presence of a feeder layer are presented separately because they were analysed separately. In addition, dose response curves showing the mean relative cloning efficiency and mean mutant frequency at each EMS dose are given for each LCL in the absence and presence of a feeder layer.

These results indicate that RS+ LCLs may be hypomutable by EMS relative to sex-matched control LCLs but RS- and FA complementation group A LCLs are normally mutable.

3.2.3 RS+ x Fanconi anaemia complementation studies

The purpose of these somatic cell hybridization

experiments was to determine if a genetic association exists between our RS+ patients and any of the four FA complementation groups. The results of the RS+ x Fanconi anaemia hybridization experiments are summarized in Table 15. Two individual replicates of fusion 6 (LB-1-*hygro* x HSC-72N [FA complementation group A]) were performed. Three HCLs were isolated from each replicate. Fusion 7 (LB-1-*hygro* x HSC-230N [FA complementation group B]) were repeated four times. The first replicate yielded no HCLs but two, three and one HCLs were isolated from the second, third and fourth replicates, respectively. The two replicates of fusion 8 (LB-1-*hygro* x HSC-536N [FA complementation group C]) yielded seven and eight HCLs for the first and second replicate, respectively. Finally, fusion 9 (LB-1-*hygro* x HSC-62N [FA complementation group D]) was repeated twice and two HCLs were isolated from each replicate. As with previous hybridization experiments, one control hybridization was performed for each parental LCL in each experiment. None of the control fusions yielded any HCLs. For cytogenetic and mitomycin C sensitivity analyses, five HCLs from fusion 6, four HCLs from fusion 7, three HCLs from fusion 8 and two HCLs from fusion 9 were selected. With the exception of fusion 9, HCLs from all successful replicates were analysed. In the case of fusion 9, the HCLs from the second replicate failed to thrive after isolation and could not be analysed so both HCLs from the first

Table 15: Results of the RS+ x Fanconi Anaemia Hybridization Experiments

Fusion Number	Parental Cell Lines	Number of Replicates	Number of HCLs Isolated	Number of HCLs Analyzed
Fusion 6	LB-1-hygro x HSC-72N	2	6	5
Fusion 7	LB-1-hygro x HSC-230N	4	6	4
Fusion 8	LB-1-hygro x HSC-536N	2	15	3
Fusion 9	LB-1-hygro x HSC-62N	2	4	2

replicate were used in the subsequent analyses. All of the HCLs analysed were shown by VNTR analysis with the probe D4139 to be true hybrids (data not shown).

Two types of cytogenetic analyses were performed on the parental LCls and the HCLs from the RS+ x Fanconi anaemia hybridization studies. First, as with the previous experiments, the cell lines were scored for the presence or absence of the RS effect. Second, the number of chromosome aberrations induced by a low, non-toxic dose of DEB was determined for each parental LCL and for at least two HCLs from each fusion. Three HCLs were tested from fusion 6.

The results of the analyses of the RS effect are presented in Table 16. All parental LCLs were determined to be hyperdiploid. Four of the five HCLs from fusion 6 and all of the HCLs from fusions 7, 8 and 9 were hypertetraploid. The fifth HCL from fusion 6 was hypotetraploid. As indicated in Table 16, 100% of the cells scored for the RS+ LCL, LB-1-hygro, exhibited the RS effect while none of the cells scored for the FA LCLs showed the abnormality. In addition, 13/14 of the HCLs failed to show the RS effect in any of their cells. A single cell from one of the hybrids from fusion 6 was scored as RS+. Chi-square analyses demonstrated that the proportion of affected cells for LB-1-hygro was highly significantly greater than those for HSC-3TO, all of the FA LCLs and all of the HCLs ($p < 0.0001$). The appearance of the chromosomes from one of

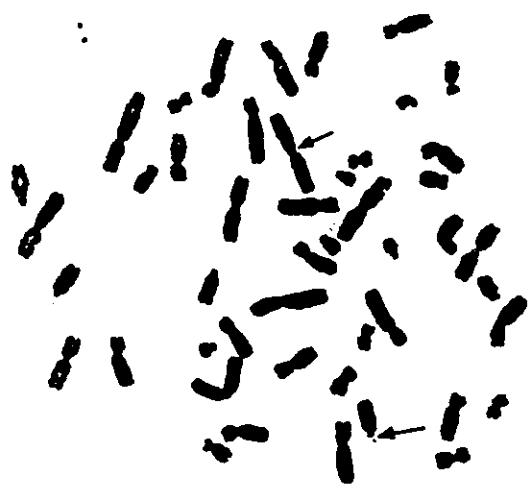
Table 16: Results of Cytogenetic Analyses for the RS Effect for RS+ x Fanconi Anaemia Hybridization Experiments

Cell Line	Modal Chromosome Number (min-max)	Number of Cells Scored	Cells with RS Effect (%)
LB-1-*hygro*	52 (46 - 54)	25	100
HSC-72N (FAA)	48 (44 - 51)	25	0
HSC-230N (FAB)	46 (44 - 50)	25	0
HSC-536N (FAC)	49 (44 - 53)	25	0
HSC-62N (FAD)	46 (43 - 47)	25	0
LB1x72N HCL 1	102 (96 - 105)	25	0
LB1x72N HCL 2	101 (89 - 105)	25	0
LB1x72N HCL 3	96 (90 - 97)	25	0
LB1x72N HCL 4	87 (77 - 93)	25	0
LB1x72N HCL 5	105 (89 - 106)	25	4
LB1x230N HCL 1	97 (95 - 99)	25	0
LB1x230N HCL 2	98 (89 - 104)	25	0
LB1x230N HCL 3	98 (89 - 104)	25	0
LB1x230N HCL 4	97 (88 - 100)	25	0
LB1x536N HCL 1	100 (93 - 105)	25	0
LB1x536N HCL 2	102 (95 - 104)	25	0
LB1x536N HCL 3	100 (92 - 104)	25	0
LB1x62N HCL 1	92 (91 - 100)	25	0
LB1x62N HCL 2	96 (91 - 99)	25	0

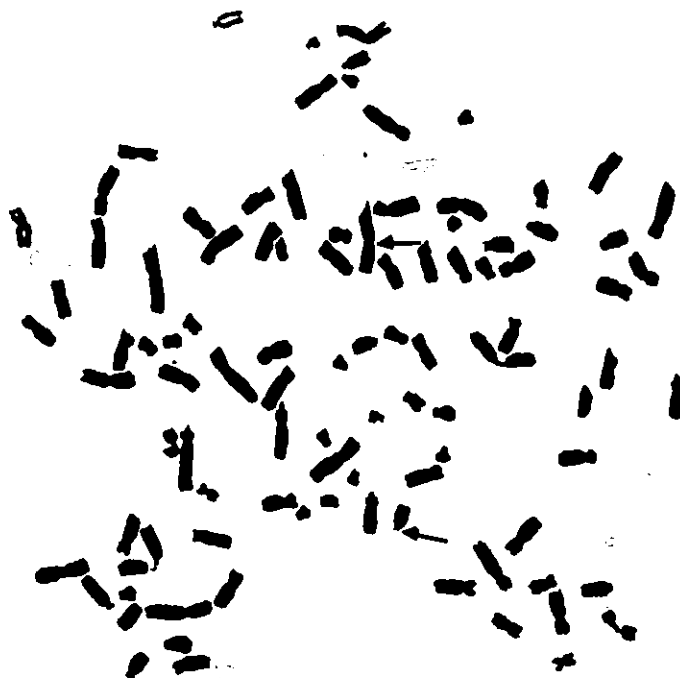
the FA LCLs and one of the HCLs is demonstrated in Figure 4.

The results of the induction of chromosome aberrations by DEB are given in Table 17. These data were analysed statistically in two ways: first, the percentages of cells with and without chromosome aberrations (minus chromatid and chromosome gaps) were compared between cell lines using Chi-square analysis and second, the mean number of chromosome aberrations per cell excluding chromatid and chromosome gaps were compared between cell lines using two-sample Student's t-tests. With respect to the Chi-square analysis, the percentage of cells with chromosome aberrations in HSC-3TO was not significantly different from that of LB-1-hygro ($p = 0.1382$). A significantly higher percentage of cells with chromosome aberrations than the control were observed in all four of the FA LCLs ($p < 0.0001$ in all cases). LB-1-hygro was also found to have a significantly lower percentage of cells with chromosome aberrations than did the four FA LCLs ($p < 0.0001$ for FA A, B and C and $p = 0.0116$ for FA D). Referring to the HCLs, the percentage of cells with chromosome aberrations was not significantly different from that of HSC-3TO in two of the three HCLs from fusion 6 and from both hybrids from each of fusions 7 and 9 (all $p \geq 0.0593$ in all cases). In contrast, LB1x72N HCL 5 had a significantly higher percentage of cells with chromosome aberrations than the control ($p = 0.0001$) as did both of the HCLs from fusion 8 ($p = 0.0032$ and

Figure 4: The appearance of the constitutive heterochromatic regions for the FA LCLs and the RS+xFA HCLs. The appearance of chromosome 1 and a D group chromosome are indicated by arrows in each photograph. A. Metaphase spread from HSC-536N; B. Metaphase spread from LB1x536N HCL 2.



A



B

Table 17: Results of Cytogenetic Analyses for DEB-induced Chromosome Aberrations for RS+ x Fanconi Anaemia Hybridization Experiments

Cell Line (n = 50)	c.a./ cell + ctg (%)*	c.a./ cell - ctg (%)	Total ctb (%)	Total ctex (%)	Total csb (%)	Total dic (%)
HSC-3TO	0.12 (12)	0.06 (6)	3 (6)	0 (0)	0 (0)	0 (0)
LB-1-<u>hygro</u>	0.16 (14)	0.14 (12)	7 (12)	0 (0)	0 (0)	0 (0)
HSC-72N	4.82 (96)	4.34 (96)	144 (82)	71 (80)	2 (4)	0 (0)
HSC-230N	7.64 (98)	7.42 (98)	261 (90)	106 (80)	0 (0)	2 (4)
HSC-536N	4.62 (96)	4.46 (96)	194 (88)	25 (38)	3 (6)	0 (0)
HSC-62N	0.46 (34)	0.36 (26)	14 (18)	2 (4)	1 (2)	0 (0)
LB1x72N HCL 2	0.36 (14)	0.14 (12)	4 (8)	0 (0)	2 (4)	1 (2)
LB1x72N HCL 4	0.18 (18)	0.14 (14)	5 (10)	1 (2)	1 (2)	0 (0)
LB1x72N HCL 5	0.76 (42)	0.50 (26)	15 (22)	3 (6)	4 (4)	3 (4)
LB1x230N HCL 2	0.08 (8)	0.08 (8)	2 (4)	1 (2)	0 (0)	1 (2)
LB1x230N HCL 3	0.06 (6)	0.02 (2)	1 (2)	0 (0)	0 (0)	0 (0)
LB1x536N HCL 1	0.24 (22)	0.20 (20)	7 (14)	2 (4)	0 (0)	1 (2)
LB1x536N HCL 3	0.46 (32)	0.30 (22)	8 (14)	7 (8)	0 (0)	0 (0)
LB1x62N HCL 1	0.14 (14)	0.12 (12)	5 (10)	0 (0)	0 (0)	1 (2)
LB1x62N HCL 2	0.08 (8)	0.04 (4)	2 (4)	0 (0)	0 (0)	0 (0)

c.a. = chromosome aberration; ctg = chromatid gap; ctb = chromatid break; ctex = chromatid exchange; csb = chromosome break; dic = dicentric chromosome; * = percentage affected cells

p = 0.0011 for HCLs 1 and 3, respectively). Similarly, LB1x72N HCL 5 had a significantly higher percentage of cells with chromosome aberrations than LB-1-hygro (p = 0.0116) while the other two HCLs from fusion 6 were not significantly different from LB-1-hygro (p = 1.0000 and p = 0.6740 for HCLs 1 and 4, respectively). With respect to the HCLs from fusion 7, LB1x230N HCL 2 was not significantly different from LB-1-hygro (p = 0.3457) while LB1x230N HCL 3 had significantly fewer cells with chromosome aberrations (p = 0.0056). Neither of the HCLs from fusion 8 were found to be significantly different from the RS+ LCL (p = 0.1228 and p = 0.0598 for HCLs 1 and 3, respectively). LB1x62N HCL 1 from fusion 9 was not significantly different from LB-1-hygro (p = 1.0000) while LB1x62N HCL 2 had a significantly lower percentage of cells with chromosome aberrations (p = 0.0371). In all cases, the FA LCLs had a significantly higher percentage of cells with chromosome aberrations than did the corresponding HCLs (p < 0.0001 for all HCLs from fusions 6, 7 and 8 and p = 0.0116 and p < 0.0001 for HCLs 1 and 2 from fusion 9, respectively).

The results of the two-sample Student's t-tests gave a similar but not identical pattern of differences between the cell lines. LB-1-hygro had significantly fewer aberrations per cell than the FA LCLs from complementation groups A, B and C (p < 0.0001) but was not significantly different from HSC-62N (complementation group D;

p = 0.1332). No significant differences were found between LB-1-hygro and any of the HCLs from fusions 7, 8 and 9 ($p \geq 0.0522$ in all cases). For fusion 9, HSC-62N was found to have significantly more aberrations per cell than LB1x62N HCL 2 ($p = 0.0226$) but not significantly more than LB1x62N HCL 1 ($p = 0.0942$).

The appearance of the chromosomes of various cell lines following DEB treatment is demonstrated in Figure 5. Figure 5A shows multiple chromatid breaks and exchanges in a cell from an FA parental LCL, HSC-230N, while Figure 5B shows the RS effect and a single chromatid break in a cell from the RS+ parental LCL, LB-1-hygro. Figure 5C shows a cell with no aberrations from an HCL which was corrected, LB1x230N HCL 3, while Figure 5D shows a cell with a few chromatid breaks and exchanges from an HCL which was not completely corrected, LB1x536N HCL 3.The mean calculated D_{10} values for each of the cell lines in fusion 6 are given in Table 18. In addition, the mean DRF for each cell line and for each class of cell lines is given. The five HCLs analysed were tested in two separate sets of experiments. As a result, the analyses for each set of experiments were done separately. For the first set of experiments, it was found that cell line had a highly significant effect on the D_{10} values ($p < 0.0001$) while experiment number had a significant effect ($p = 0.0189$). The effect of replicate number was found to be non-significant ($p = 0.4893$). For

Figure 5: Chromosome appearance following DEB treatment. Chromosome aberrations are indicated by arrows in each photograph. A. Metaphase spread from HSC-230N; B. Metaphase spread from LB-1-*hygro*.

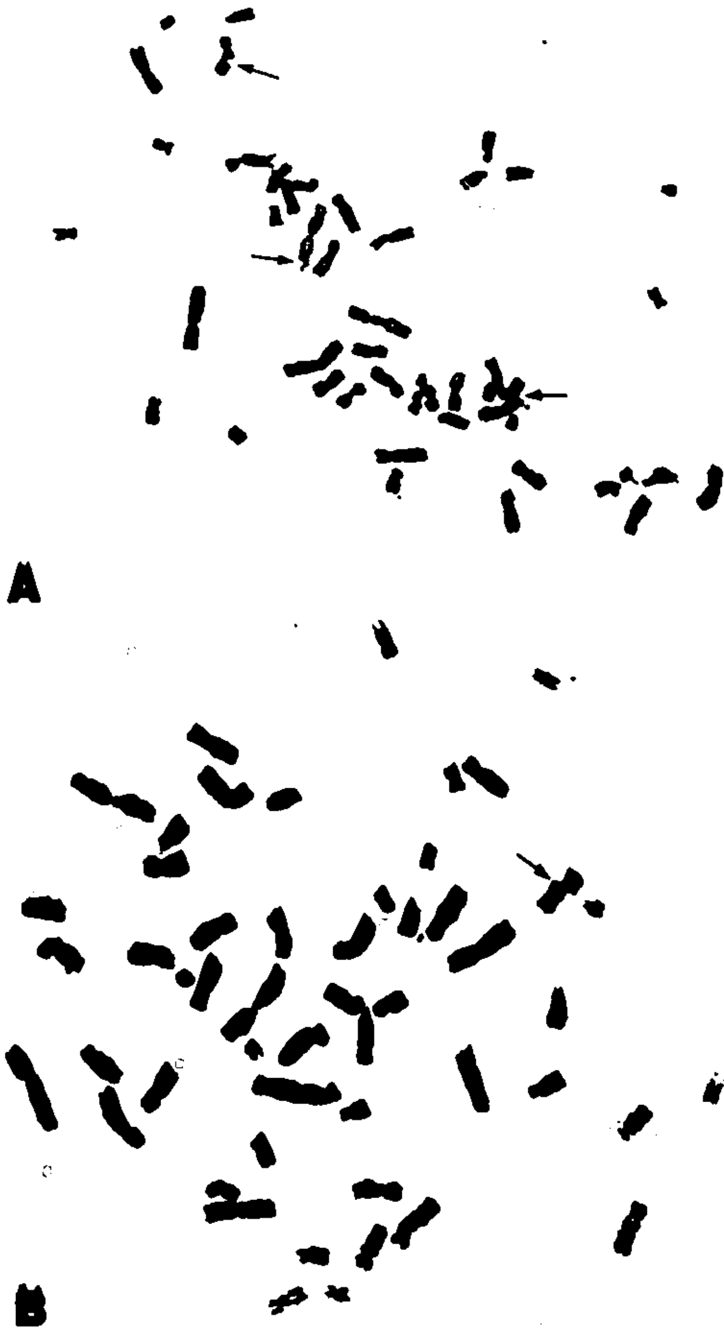

A
B

Figure 5 (cont'd): Chromosome appearance following DEB treatment. C. Metaphase spread from LB1x230N HCL 2; D. Metaphase spread from LB1x536N HCL 3.

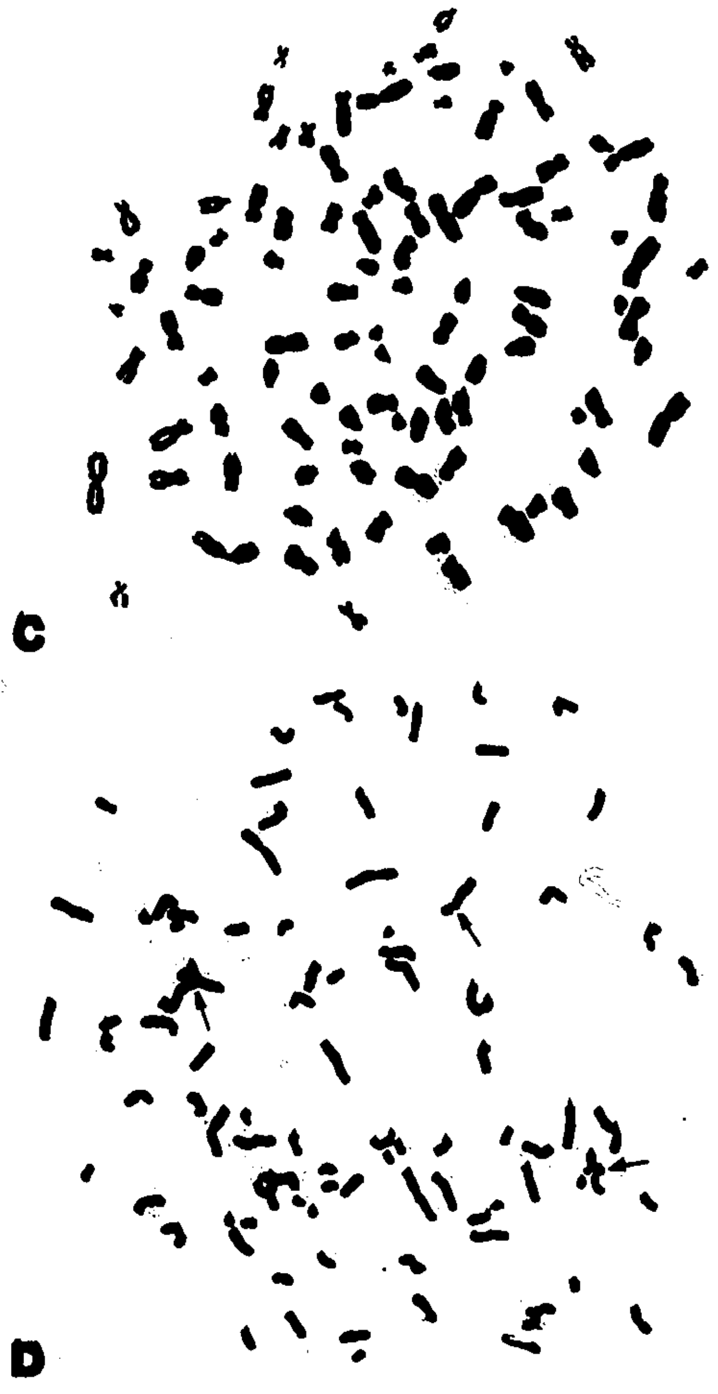

C
D

Table 18: Mean Fitted Mitomycin C D_{10} Values and Dose Reduction Factors for RS+ x FA A Hybridization Experiments

Cell Line	Mean D_{10}* (sd) (ng/mL)	Mean DRF** (sd)	Mean DRF for class (sd)	P value for mean = 1.00
HSC-3TO	141.24 (62.55)	1.00	1.00	1.0000
LB-1 -hygro	58.77 (9.17)	2.84 (0.89)	2.84 (0.89)	<0.0001
HSC-72N	3.08 (2.90)	97.00 (94.00)	97.00 (94.00)	0.0010
LB1x72N HCL 1	92.40 (35.90)	1.59 (0.56)	2.73 (2.02)	<0.0001
LB1x72N HCL 2	63.93 (38.40)	2.48 (1.91)		
LB1x72N HCL 3	60.64 (13.79)	2.26 (1.11)		
LB1x72N HCL 4	28.07 (5.93)	5.04 (2.14)		
LB1x72N HCL 5	118.00 (52.17)	1.71 (1.43)		

* D_{10} = the mitomycin C dose at which 10% survival is estimated to occur from fitted regression equation

** Dose Reduction Factor (DRF) = $\frac{D_{10} \text{ HSC-3TO}}{D_{10} \text{ cell line}}$

the second set of experiments, the effect of cell line was again found to be highly significant ($p < 0.0001$) while the effects of experiment number and replicate number were not significant ($p = 0.9019$ and $p = 0.9768$, respectively).

The Tukey's WSD pairwise comparisons produced some equivocal results. In the first set of experiments, it was found that, at $\alpha = 0.05$, HSC-3TO, LB-1-*hygro* and LB1x72N HCL 1 all had mean D_{10} values that were significantly different from each other and from those of each of the other cell lines tested. HSC-72N and LB1x72N HCL 4 were found to have mean D_{10} values that were not significantly different from each other despite a difference of more than 8-fold. Thus, a type II statistical error is probably occurring here as a result of the low power of the comparison. A two-sample Student's t-test between these two cell lines supports this hypothesis by showing a highly significant difference between their mean D_{10} values ($p < 0.0001$). Comparisons between the cell lines tested in the second set of experiments showed that HSC-3TO, LB1x72N HCL 2 and LB1x72N HCL 5 had mean D_{10} values that were not significantly different from each other. In addition, LB-1-*hygro* and LB1x72N HCL 3 had mean D_{10} values that were neither significantly different from each other nor significantly different from those of the other two HCLs and that of HSC-72N. HSC-72N had a mean D_{10} value that was significantly lower than those of HSC-3TO, LB1x72N HCL 2 and

LB1x72N HCL 5. As mentioned previously, these equivocal results may be due to a lack of statistical power. Accordingly, two-sample Student's t-tests were performed on all pairs of cell lines shown not to be significantly different. HSC-3TO and LB1x72N HCL 5 had mean D_{10} values that were not significantly different from each other ($p = 0.3514$) but HSC-3TO was found to be significantly less sensitive than LB1x72N HCLs 2 and 3 ($p= 0.0016$ and $p = 0.0001$, respectively). Similarly, LB1x72N HCL 5 was significantly less sensitive than LB1x72N HCL 3 ($p = 0.0196$) and not significantly different from LB1x72N HCL 2 ($p = 0.0576$). The mean D_{10} values of HCLs 2 and 3 were not significantly different from each other ($p = 0.3344$) nor were they significantly different from that of LB-1-*hygro* ($p = 0.2482$ and $p = 0.7330$, respectively). LB-1-*hygro*, was, however, significantly more sensitive than both HSC-3TO and LB1x72N HCL 5 ($p = 0.0001$ and $p = 0.0154$, respectively). Finally, HSC-72N was significantly more sensitive than all of the other cell lines ($p \leq 0.0004$, in all cases). Thus, to summarize the results of all the statistical analyses for the cell lines from fusion 6, HCL 5 appears to have been corrected with respect to MMC sensitivity to the level of the control cell line, HSC-3TO while HCLs 2 and 3 have been corrected only to the level of the RS+ cell line, LB-1-*hygro*. HCL 1 has been corrected to a level between those of the control and RS+ cell lines and HCL 4 has been

corrected to a level between those of the RS+ and FA cell lines.

The mean DRF for the RS+ LCL, LB-1-hygro (2.86) was significantly different from the expected value of 1.00 ($p < 0.0001$) as was the mean DRF for the FA LCL, HSC-72N (96.97; $p = 0.0010$). In addition, the mean DRF for the LB1x72N HCLs (2.73) was significantly higher than 1.00 ($p < 0.0001$) and significantly different from that of HSC-72N ($p = 0.0011$) but not significantly different from that of LB-1-hygro ($p = 0.7356$).

The mean fitted D_{10} values and DRFs for the parental LCLs and HCLs from fusion 7 are given in Table 19. As with fusion 6, the hybrids for fusion 7 were tested in two separate sets of experiments. In the first set of experiments, the effect of cell line on the D_{10} values was found to be highly significant ($p < 0.0001$) while those of experiment number and replicate number were not significant ($p = 0.5152$ and $p = 0.7907$, respectively). For the second set of experiments, the effect of cell line was once again highly significant ($p < 0.0001$) but the effect of experiment number was also found to be significant ($p = 0.0053$). The effect of replicate number was not significant ($p = 0.3399$).

The mean D_{10} value of HSC-3TO was not significantly different from that of LB1x230N HCL 1 in the first set of experiments nor was it significantly different for those of LB1x230N HCLs 2, 3 and 4 in the second set of experiments.

Table 19: Mean Fitted Mitomycin C D_{10} Values and Dose Reduction Factors for RS+ x FA B Hybridization Experiments

Cell Line	Mean D_{10}* (sd) (ng/mL)	Mean DRF** (sd)	Mean DRF for class (sd)	P value for mean = 1.00
HSC-3TO	112.37 (25.82)	1.00	1.00	1.0000
LB-1 -hygro	45.93 (18.38)	2.83 (1.19)	2.83 (1.19)	0.0002
HSC-230N	5.16 (1.96)	25.56 (13.65)	25.56 (13.65)	<0.0001
LB1x230N HCL 1	95.55 (25.11)	1.29 (0.56)	1.23 (0.46)	0.0081
LB1x230N HCL 2	99.89 (46.73)	1.26 (0.41)		
LB1x230N HCL 3	124.12 (45.24)	1.00 (0.44)		
LB1x230N HCL 4	88.17 (18.39)	1.37 (0.42)		

* D_{10} = the mitomycin C dose at which 10% survival is estimated to occur from fitted regression equation

** Dose Reduction Factor (DRF) = $\frac{D_{10}\ \text{HSC-3TO}}{D_{10}\ \text{cell line}}$

Similarly, in both sets of experiments the mean D_{10} values of LB-1-*hygro* were significantly lower than those of the HCLs and HSC-3TO. The mean D_{10} values of HSC-230N were significantly lower than those of the HCLs and HSC-3TO in both analyses as well as being significantly lower than that of LB-1-*hygro* in the first set of experiments ($\alpha = 0.05$).

The mean DRF for LB-1-*hygro* (2.83) was significantly different from 1.00 ($p = 0.0002$). Similarly, the mean DRF for the FA LCL, HSC-230N (25.56), was significantly higher than expected ($p < 0.0001$). The mean DRF for the LB1x230N HCLs (1.23) was also significantly higher than 1.00 ($p = 0.0081$) as well as being significantly lower than those for both LB-1-*hygro* and HSC-230N ($p = 0.0007$ and $p < 0.0001$, respectively).

Table 20 presents the mean calculated D_{10} values and DRFs for the cell lines from fusion 8. Of the three factors tested in the analysis of variance, only cell line was found to exert a significant effect on the D_{10} values ($p < 0.0001$) while those of experiment number and replicate number were not significant ($p = 0.8218$ and $p = 0.8143$, respectively). The mean D_{10} value of HSC-3TO was found to be significantly higher than those of all of the other cell lines tested. Similarly, the mean D_{10} value of LB-1-*hygro* was significantly higher than those of all three LB1x536N HCLs and that of HSC-536N. The mean D_{10} values of the three HCLs were not significantly different from each other but

Table 20: Mean Fitted Mitomycin C D_{10} Values and Dose Reduction Factors for RS+ x FA C Hybridization Experiments

Cell Line	Mean D_{10}* (sd) (ng/mL)	Mean DRF** (sd)	Mean DRF for class (sd)	P value for mean = 1.00
HSC-3TO	106.28 (15.23)	1.00	1.00	1.0000
LB-1 -hygro	52.58 (14.27)	2.21 (0.74)	2.21 (0.74)	0.0001
HSC-536N	1.17 (0.80)	112.60 (47.79)	112.60 (47.79)	<0.0001
LB1x536N HCL 1	27.64 (7.39)	4.01 (0.90)	3.36 (0.87)	<0.0001
LB1x536N HCL 2	35.67 (4.14)	3.03 (0.67)		
LB1x536N HCL 3	37.88 (6.67)	3.04 (0.69)		

* D_{10} = the mitomycin C dose at which 10% survival is estimated to occur from fitted regression equation

** Dose Reduction Factor (DRF) = $\frac{D_{10} \text{ HSC-3TO}}{D_{10} \text{ cell line}}$

were significantly higher than that of HSC-536N ($\alpha = 0.05$).

The mean DRF for LB-1-hygro (2.16) was once again found to be significantly higher than 1.00 ($p = 0.0001$) as was the mean DRF for the FA LCL, HSC-536N (112.65; $p < 0.0001$). The mean DRF for the LB1x536N HCLs (3.36) was also significantly higher than 1.00 ($p < 0.0001$) and than the mean DRF for LB-1-hygro ($p = 0.0001$). The mean DRF for HSC-536N was significantly higher than that of the HCLs ($p < 0.0001$).

The mean calculated D_{10} values and DRFs for the parental LCLs and HCLs from fusion 9 are presented in Table 21. The effects of both cell line and experiment number on the calculated D_{10} value were found to be highly significant ($p < 0.0001$ and $p = 0.0001$, respectively) while the effect of replicate number was not significant ($p = 0.5171$). HSC-3TO had a mean D_{10} value that was significantly higher than all of the other cell lines tested. Similarly, HSC-62N had a mean D_{10} value significantly lower than those of all of the other cell lines. The mean D_{10} values of LB-1-hygro and the two LB1x62N HCLs were not significantly different from each other ($\alpha = 0.05$).

The mean DRF for LB-1-hygro (2.01) was significantly higher than 1.00 ($p < 0.0001$) as was the mean DRF for the FA LCL, HSC-62N (12.46; $p < 0.0001$). In addition, the mean DRF for the LB1x62N HCLs (1.77) was significantly higher than

Table 21: Mean Fitted Mitomycin C D_{10} Values and Dose Reduction Factors for RS+ x FA D Hybridization Experiments

Cell Line	Mean D_{10}* (sd) (ng/mL)	Mean DRF** (sd)	Mean DRF for class (sd)	P value for mean = 1.00
HSC-3TO	101.41 (13.33)	1.00	1.00	1.0000
LB-1 -hygro	51.89 (11.85)	2.01 (0.38)	2.01 (0.38)	<0.0001
HSC-62N	9.63 (4.11)	12.46 (5.49)	12.46 (5.49)	<0.0001
LB1x62N HCL 1	67.42 (29.10)	1.68 (0.54)	1.77 (0.48)	<0.0001
LB1x62N HCL 2	57.75 (11.83)	1.91 (0.37)		

* D_{10} = the mitomycin C dose at which 10% survival is estimated to occur from fitted regression equation

** Dose Reduction Factor (DRF) = $\frac{D_{10}\ \text{HSC-3TO}}{D_{10}\ \text{cell line}}$

the expected value ($p < 0.0001$). The mean DRFs for LB-1-hygro and the LB1x62N HCLs were not significantly different from each other ($p = 0.1304$) but both were significantly lower than that of HSC-62N ($p < 0.0001$ in both cases).

The results of the cytogenetic and MMC sensitivity analyses suggest that LB-1-hygro and HSC-230N (FA complementation group B) belong to different complementation groups. Incomplete correction of MMC hypersensitivity in hybrids from fusions 6 and 9 may be indicative of a genetic association between LB-1-hygro and FA LCLs from complementation groups A and D. In addition, incomplete correction of both the chromosomal sensitivity to DEB and MMC hypersensitivity in hybrids from fusion 8 suggests an association between RS+ LCLs and FA complementation group C LCLs.

4. DISCUSSION

4.1 Genetic Heterogeneity in Roberts Syndrome

4.1.1 Association between the RS effect and hypersensitivity to mitomycin C in RS+ cells

Fibroblasts from RS patients exhibiting the RS effect have been demonstrated to be hypersensitive to cell killing by a number of mutagenic agents including mitomycin C (MMC), ultraviolet irradiation, cisplatin, 8-methoxypsoralen and methylnitrosourea relative to control fibroblasts (Gentner et al., 1985; 1986; Burns and Tomkins, 1989). A corresponding hypersensitivity to mitomycin C was not seen in either RS- fibroblasts or fibroblasts from RS+ obligate heterozygotes (Burns and Tomkins, 1989). The present study used lymphoblastoid cell lines (LCLs) instead of fibroblasts and thus, it was first necessary to demonstrate the mitomycin C hypersensitivity in this cell type. It was found that RS+ LCLs from two different patients were 2- to 3-fold more sensitive to cell killing by MMC than were control or RS- LCLs. The sensitivities of the control and RS- LCLs were not significantly different from each other.

Hybridization studies between control and RS+ LCLs were designed to answer the question of whether or not both

the RS effect and the MMC hypersensitivity cellular phenotypes could be corrected in somatic cell hybrids. The correction of the RS effect in intra- and inter-species somatic cell hybrids between RS+ fibroblasts and normal cells has been demonstrated previously (Krassikoff et al., 1986; Knoll and Ray, 1986; Gunby et al., 1987). It was found that the RS+ x control hybrid cell lines (HCLs) were completely corrected with respect to both the cytogenetic abnormality and the MMC hypersensitivity characteristics of RS+ LCLs. Therefore, both of these cellular phenotypes are recessive characteristics, as has been previously suggested (Gunby et al., 1987; Burns and Tomkins, 1989). In addition, these observations strengthen the possibility of an association between the RS effect or heterochromatin structure and function and hypersensitivity to MMC.

The basis of the association between the RS effect and MMC hypersensitivity is unclear. It has been suggested in Drosophila melanogaster that a defective allele controlling the mitotic condensation of heterochromatin but not euchromatin at the mus-101 locus may render the chromatin more susceptible to DNA damage by mutagens as a direct result of a disruption in the organization of heterochromatin. Alternatively, the abnormal heterochromatin condensation may prevent the normal repair of mutagen-induced lesions (Gatti et al., 1983). It is possible that a similar phenomenon is occurring in RS+

cells. That is, the primary defect of abnormal heterochromatin structure has the secondary effect of rendering the chromatin either more susceptible to damage by mutagens or less accessible to DNA repair enzymes.

An alternative hypothesis is that RS+ cells are defective in a process of DNA repair, perhaps that of the repair of the intra- and inter-strand crosslinks induced by MMC (Burns and Tomkins, 1989). It has been found that MMC is an extremely potent inducer of sister chromatid exchanges (SCEs) in human lymphocytes even at very low doses (Latt, 1974). RS+ LCLs treated with MMC have significantly fewer induced SCEs compared to either treated control LCLs or RS- LCLs (Burns and Tomkins, 1987). Given the hypersensitivity to MMC exhibited by RS+ fibroblasts, it was suggested that the RS+ LCLs are deficient in the repair of the crosslinks induced by MMC and only those cells with relatively little damage survive treatment.

Finally, it is possible to envisage a mechanism whereby the hypomethylation of DNA in RS+ cells may contribute to its hypersensitivity to MMC. Mitomycin C intercalates between two deoxyguanylate (dG) residues to generate inter-strand crosslinks (Tomasz _et al._, 1987). This crosslink must occur by necessity between two CpG dinucleotides on opposite strands. Stereochemical studies have shown that the MMC molecule fits extremely snugly into the DNA structure (_ibid._). It could be speculated that if

the deoxycytidylate (dC) molecules at the CpG dinucleotides were methylated, as they are in 50 - 70% of cases in various vertebrate species (Wigler, 1981), it may not be possible for the crosslink to be formed. Crosslinks would only occur, therefore, at unmethylated CpG dinucleotides. It has been shown that DNA from RS+ cells is hypomethylated compared to DNA from normal or RS- cells (Harrison and Tomkins, 1991a). This lower level of methylation may permit the formation of a higher number of MMC-induced crosslinks resulting in an increased sensitivity of RS+ cells to MMC treatment.

4.1.2 Genetic heterogeneity in Roberts syndrome

Genetic heterogeneity has been demonstrated in a number of conditions thought to involve defects in DNA repair including xeroderma pigmentosum (XP), ataxia telangiectasia (AT) and Fanconi anaemia (FA) (Timme and Moses, 1988; Strathdee et al., 1992a). It is not clear, however, whether different complementation groups from each of these diseases present distinct clinical pictures or whether the range of clinical symptoms is the same for all complementation groups (McKusick, 1990, XP: 278700, AT:208900). In each of these diseases, the complementation groups were delineated using somatic cell hybridization and the subsequent examination of hybrids for correction of mutagen hypersensitivity cellular phenotypes (Robbins et

al., 1974; Jaspers and Bootsma, 1982; Strathdee et al., 1992a). The presence of genetic heterogeneity in conditions involving defects of DNA repair has not been universal, however. Recently, it has been reported that Bloom syndrome (BS) patients of diverse ethnic origin are defined by a single complementation group (Weksburg et al., 1988).

The wide range of clinical heterogeneity in Roberts syndrome patients is suggestive of a corresponding genetic heterogeneity. That is, patients belonging to the same complementation group may present with similar clinical manifestations while those patients from different complementation groups may be more heterogeneous. Given that the two sub-groups of patients in RS, RS+ and RS-, are clinically indistinguishable and that the clinical heterogeneity within each sub-group is as great as that for all RS patients, it was desirable to determine if genetic heterogeneity was present both between different RS+ patients and between RS+ and RS- patients. It was not possible to assess genetic heterogeneity between different RS- using somatic cell hybridization because an abnormal cellular phenotype for RS- cells has not been identified.

The evidence presented suggests that the two RS+ patients examined possess the same genetic defect and thus, belong to the same complementation group. In both the cytogenetic analyses and the MMC sensitivity analyses, the R20xLB1 HCLs failed to exhibit correction of the cellular

defects unique to RS+ cells. In fact, one of the HCLs, HCL 2, was found to be more severely compromised with respect to MMC sensitivity than was one of the parental LCLs, LB-1-hygro. It is premature at this point to extrapolate these results to include all RS+ patients. However, the two patients tested here originate from two extremely diverse ethnic groups, LB-1 is from a Manitoba Mennonite population and R20 is from an isolated French Canadian population, and it is tempting to conclude that the level of genetic heterogeneity between RS+ patients may be quite low.

It is difficult to envisage given the extremely low incidence of RS that the defect in both of these populations arose from a mutation in a common ancestor. That is, given the ethnic diversity of the two populations, one would have to trace back many generations to find a common link. If this was the case, the incidence of the mutant allele in the populations would be expected to be relatively high and the incidence of RS correspondingly more common. This is not the case. The incidence of RS is very low even in these relatively inbred populations. Thus, the mutant alleles in the two populations likely arose independently. Despite the expectation that the independent mutations could involve different genes, patients from the two populations belong to the same complementation group.

The hybridization experiments between RS- and RS+ LCLs demonstrated that RS- cells are capable of

complementing both of the cellular defects unique to RS+ cells. In both fusion 4 and fusion 5, complete correction of both the RS effect and the MMC hypersensitivity of RS+ cells was observed. These data exclude the first model of inheritance for RS which suggests no genetic heterogeneity between RS+ and RS- patients. The normal heterochromatin appearance and normal MMC response of RS- cells are dominant to the abnormalities in RS+ cells which suggests different defects in the two sub-groups of patients. It was not possible from these data to determine whether RS+ and RS- patients arise from allelic mutations or mutations in separate genes. The only definitive way of distinguishing between these two possibilities would be the cloning of the RS+ gene and subsequent examination of the same gene in RS- cells to determine what, if any, mutations are present. The differences at the cellular level between RS+ and RS- cells are more easily explained, however, by a model of genetic heterogeneity rather than one of allelic heterogeneity. If the cellular abnormalities characteristic of RS+ cells were simply caused by mutations in different domains of the RBS gene than those affected in RS- cells, one would expect to occasionally find patients exhibiting one of the defects but not others because their mutations do not affect all of the critical domains. No such findings have been reported to date.

An alternative model for the relationship between

RS+ and RS- patients is that of co-recessive inheritance (C-RI) (Lambert and Lambert, 1985; 1989; 1992). This model predicts that an individual must be homozygous or hemizygous for defective alleles at two or more genetic loci in order for a condition to be expressed. Originally suggested as an explanation for the large number of complementation groups identified in XP despite a biochemically limited associated DNA repair defect (Lambert and Lambert, 1989), C-RI can also be used to explain other diseases defective in DNA repair such as AT and FA. It could be suggested that RS+ and RS- patients are examples of C-RI which share a common recessive defect. Each subgroup would also possess unique recessive defects that would account for the differences between them.

The results of the hybridization studies between the two RS+ LCLs suggest the possibility of a low level of genetic heterogeneity between RS+ patients. It will be important to test this hypothesis by fusing LCLs from patients with a variety of ethnic backgrounds to each other and examining the hybrids for complementation of the RS effect and mutagen hypersensitivity cellular defects. It may also be informative to study the hybrids for correction of the other cellular defects reported in RS+ cells such as mitotic abnormalities, abnormal interphase nuclear morphology and abnormal replication pattern (Tomkins and Sisken, 1984; Jabs et al., 1991, Harrison and Tomkins, 1991b) to determine if these defects are also recessive and

if their association with the RS effect is maintained in hybrids.

The most definitive way to distinguish between the models of allelic heterogeneity and genetic heterogeneity between RS+ and RS- patients will be to clone the RS+ gene and determine if any mutations are present in that gene in RS- cells. The most promising approach to cloning the RBS gene is functional complementation (Strathdee et al., 1992b). This method involves transfecting the cells with a cDNA expression library constructed in Epstein-Barr virus (EBV)-based shuttle vectors and then selecting for cells capable of surviving several rounds of treatment with mutagens to which the cells are known to be hypersensitive. The episomally maintained vectors can then be recovered from surviving cells and characterized (ibid.). Before this is possible, however, it will be necessary to identify a mutagen to which RS+ LCLs are more than 5-fold hypersensitive. This level of hypersensitivity is necessary to ensure that the population of cDNA clones isolated from surviving cells following selection in the mutagen(s) is enriched for the clone containing the RBS cDNA. If a sufficient level of hypersensitivity is not present, the cells surviving treatment may not contain the RBS cDNA and a series of non-specific cDNAs will be isolated. Indeed, this was the case when isolation of the RBS gene was attempted using functional complementation of sensitivity to gamma

irradiation (Van Den Berg and Francke, 1991). In this case, more than 20 different cDNA clones were isolated from the surviving cell populations, none of which were represented more than the others (U. Francke, personal communication). Thus, it will first be important to identify at least one, preferably two, mutagens to which RS+ cells exhibit a very high level of hypersensitivity before this method of isolation of the RBS gene is attempted. If such a mutagen cannot be identified for lymphoblastoid cells, the isolation could be attempted in EBV-transformed fibroblasts since fibroblasts have been found to be more sensitive compared to controls than are lymphoblasts from the same patients. For example, fibroblasts from the same patient as the RS+ LCL, R20, used here were found to be approximately 5-fold more sensitive than controls to cell killing by MMC (Burns and Tomkins, 1989) while R20 was shown here to be approximately 2- to 3-fold hypersensitive.

4.2 Similarities Between Roberts Syndrome and Fanconi Anaemia

RS and FA share a number of clinical similarities. Both conditions are characterized by limb abnormalities often involving the radius and thumb although other skeletal abnormalities are seen in both conditions (Freeman et al., 1974; Giampietro et al., 1992). In addition, cranial anomalies, renal anomalies, cardiac defects and

genitourinary malformations have been reported in both syndromes (O'Brien and Mustard, 1921; Herrmann et al., 1969; Judge, 1973; Freeman et al., 1974; Grosse et al., 1975; Mann et al., 1982; Grundy et al., 1988; Giampietro et al., 1992). Evidence for the existence of clinical overlap between RS and FA is provided by the fact that both FA and RS+ patients have been mis-diagnosed as having Baller-Gerold syndrome (Huson et al., 1990; Van Maldergem et al., 1992; Farrell et al., 1993). Baller-Gerold syndrome is another rare, recessively-inherited condition characterized by growth retardation and limb abnormalities (Baller, 1950; Gerold, 1959). To date, there have been 12 cases of this condition reported (Van Maldergem et al., 1992). Two of these cases have been subsequently re-diagnosed as Roberts syndrome on the basis of the presence of the RS effect in the chromosomes of the patients (Huson et al., 1990; Van Maldergem, personal communication). Similarly, another patient has been recently re-diagnosed as Fanconi anaemia on the basis of elevated chromosome aberrations in response to treatment with DEB (Farrell et al., 1993). Thus, considerable clinical overlap is evident between these three conditions. In addition, the similarities between RS+ and FA cells with respect to cellular sensitivity to DNA crosslinking agents are suggestive of a possible genetic relationship between the two conditions. It has been suggested that RS and FA may be allelic (Hall, 1987).

There were three objectives to the EMS mutability portion of the research project: (1) to determine the cellular sensitivity of RS+, RS- and FA LCLs to treatment with ethyl methanesulphonate (EMS) relative to that of control LCLs as measured by cloning efficiency following treatment; (2) to determine the mutability of RS+, RS- and FA LCLs by EMS compared to that of control LCLs as measured by a clonal assay; and (3) to generate a universal hybridizing RS+ LCL for use in complementation studies. The first two of these objectives were met with some success. However, the third objective was abandoned due to the great difficulty encountered in isolating mutated RS+ cells and alternative methods were sought (see below).

4.2.1 Sensitivity of RS+ cells to EMS treatment

The RS+ LCLs were found to be approximately 2-fold more sensitive to EMS treatment than the six control LCLs, as indicated by their EC_{50} values, both individually and when the data from the two LCLs were pooled. Neither of the RS- LCLs was found to be significantly more sensitive to EMS treatment than were the control LCLs nor was a significant difference shown when the EC_{50} data from the two RS- LCLs were pooled. Finally, the pooled EC_{50} data from the two FA LCLs from complementation group A also showed a significantly more sensitive response to EMS treatment than that of the controls (approximately 1.7-fold). One of the

FA LCLs, HSC-72OT, was also found to be significantly more sensitive when it was tested alone while the other FA LCL, HSC-99, showed a non-significant difference when tested separately. These observations suggest that the mutagen sensitivity spectrum of RS+ cells is not limited to crosslinking agents but also includes alkylating agents. There are also indications that FA cells from complementation group A may have some deficiencies in repairing EMS-induced damage as well. The predominant mutation induced by EMS in human cells is a GC to AT transition as a result of mispairing of a T residue with the O^6-ethyl-guanine adducts produced by EMS (Loechler et al., 1984; Ingle and Drinkwater, 1989). Thus, if the RS+ and FA group A cells are incapable of repairing these adducts, they may accumulate to a lethal level at a much lower dose than that required to kill control cells.

The importance of these results must not be overemphasised, however, given that one of the control LCLs, DM, was more than 2.5-fold more sensitive to EMS treatment than were the other five controls. This is not an unprecedented finding in control LCLs as it has been shown that TK6 is approximately one order of magnitude more sensitive to cell killing by EMS treatment compared to other mammalian cell lines (O'Brien et al., 1990; Waters et al., 1990). Thus, it appears that there is a subset of control LCLs on which EMS has a greater cytotoxic effect than it has

on the majority of controls. However, given that only 1 of 6 of the control LCLs used in these studies and neither of the RS- LCLs exhibited this effect while both of the RS+ LCLs were hypersensitive, this may be indicative of a genuine deficiency in RS+ cells. It will be important to examine the responses of more RS+ cell lines as well as many more controls to determine if such a deficiency does exist. Certainly, this does not appear to be as important a deficiency as is the hypersensitivity of RS+ LCLs to MMC.

4.2.2 Mutability of RS+ cells by EMS

All mutant frequency comparisons between cell lines were done at the EC_{50} value for each LCL as a way of minimizing the effect of the differing dose responses between LCLs (Papadopoulo _et_ _al_. 1990a,b). In the absence of an irradiated TK6 feeder layer, RS+ LCLs were found to be hypomutable at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus relative to their sex-matched controls. Indeed, a mutant frequency of zero was obtained for all EMS doses for R20 thus making the degree of hypomutability (the mutant frequency reduction factor [MFRF]) uncalculable for this LCL (a mutant frequency of 10^{-4} or greater could have been detected based on the number of cells used and the non-selection cloning efficiency). The other RS+ LCL, LB-1 was approximately 7-fold less mutable that its sex-matched controls at its EC_{50} value.

These data, taken together with the possibly increased cellular sensitivity of RS+ LCLs to EMS, suggest that these cells may be deficient in a DNA repair pathway responsible for processing the lesions generated by EMS. That is, the accumulation of unrepaired lesions results in a lower lethal EMS dose for these cell lines so that only cells with relatively little damage will survive treatment. These cells are less likely to have mutations at the HPRT locus and thus, a lower mutant frequency is observed.

Neither of the RS- LCLs was found to be hypomutable relative to its sex-matched controls. Indeed, LoGi was significantly hypermutable while TiBu showed no significant difference. Thus, as would be expected based on their normal responses to MMC and EMS treatment, these LCLs do not appear to have any deficiencies with respect to the normal processing of EMS-induced lesions.

The FA complementation group A LCL, HSC-99, was not found to be significantly different from its controls at its EC_{50} mutant frequency value as measured by its MFRF. This finding is inconsistent with a previous report of hypomutability of FA fibroblasts by EMS (Finkelberg et al., 1977). However, as these studies were done prior to the assignment of complementation groups in FA, it is possible that cells from a complementation group other than group A were studied. As only group A cells were included in the present studies, no comment can be made about cells from the

other complementation groups. In a previous study, HSC-99 has been reported to be hypomutable in response to treatment by photoactivated psoralens (PUVA) (Papadopoulo et al., 1990a). PUVA are bifunctional crosslinking agents to which FA LCLs from complementation groups A and D have been shown to have significantly increased cellular sensitivities (Papadopoulo et al., 1990a;b). No significant difference was found between the EMS EC_{50} of HSC-99 and those of the controls in the present study. Thus, it appears that the hypomutability exhibited by HSC-99 may be directly related to its cellular sensitivity to the mutagen. It was suggested that, based on a lower efficiency of crosslink incision and of crosslinkable furan-side monoadduct repair, that HSC-99 may be deficient in an error-prone DNA repair pathway responsible for the processing of such lesions (Averbeck et al., 1988; Papadopoulo et al., 1990a). If such a deficiency does exist in this LCL, this pathway must not be involved in the processing of EMS-induced lesions as HSC-99 appears to respond normally to EMS treatment with respect to mutability.

The system used in these studies has a number of limitations that restricted its value for determining the relative mutant frequencies of different LCLs. First, extreme variability was observed between replicate experiments of the same LCL. In many cases, the standard deviation for a specific dose was as large or larger as the

mutant frequency itself. Second, the non-selection cloning efficiency obtained for all of the LCLs studied was very low, always less than 10% and in some cases, less than 1%. Because the mutant frequency is calculated by dividing the selection cloning efficiency by the non-selection cloning efficiency, the mutant frequency is inversely related to the non-selection cloning efficiency so that a very low cloning efficiency will result in a very large mutant frequency. This was especially evident with one of the control LCLs (HSC-55) which exhibited cloning efficiencies of much less than 1% over all of the doses used and, despite selection cloning efficiencies of the same order of magnitude as the other male control (DM), had huge mutant frequencies as a result. A significant negative correlation was found between the non-selection cloning efficiency and the mutant frequency both in the absence and in the presence of a feeder layer ($p > 0.05$). Thus, this confounding effect of low cloning efficiency severely limits the usefulness of these data in determining realistic mutant frequencies.

It has previously been shown that the use of a feeder layer of $HPRT^-$ cells will improve the cloning efficiency of poorly cloning cells to an acceptable level (Papadopoulo _et al_., 1990a). Given the extremely poor cloning efficiencies obtained even with the control LCLs, it was hoped that using an irradiated TK6 feeder layer would increase the cloning efficiencies. Unfortunately, the

opposite effect was observed. In all experiments when a feeder layer was used, the cloning efficiency decreased. This appeared to be a direct effect of the feeder layer because when the same culture was divided and seeded into plates with or without feeder cells, the cloning efficiency with the feeder cells was always lower than that without them, often by as much as two-fold or more. As a result extreme variability was observed between experiments and between LCLs and unreasonably large mutant frequencies were obtained especially for HSC-55, LoGi and R20. The only significant difference observed with these data between control LCLs and the other LCLs studied was a significant hypermutability in LoGi.

In summary, there is some evidence that RS+ LCLs may be hypomutable compared to sex-matched controls by EMS at the HPRT locus. No such hypomutability was observed with RS- LCLs. A previously reported hypomutability of FA fibroblasts by EMS was not observed with the FA LCL studied here. Extremely low cloning efficiencies observed with all LCLs have a confounding effect on the calculated mutant frequency values for each LCL and may limit the accuracy of the comparisons made between LCLs. The use of irradiated HPRT- feeder cells did not improve cloning efficiency in these experiments.

There is sufficient evidence of hypomutability in RS+ cells to pursue this line of research further. However,

it will be very important to improve the cloning efficiencies of all the cell lines used to a level that does not have a confounding effect on the calculation of the mutant frequency. This may be achieved in a number of ways. The feeder cells used here were irradiated TK6 cells. A previous study reported that the use of GM1899A cells, a Lesch-Nyhan LCL, improved the cloning efficiency of FA LCLs from 1 to 3% in the absence of feeder cells to 16 to 40% in the presence of feeder cells (Papadopoulo et al., 1990a). Perhaps GM1899A LCLs provide a better conditioning of the medium and thus, support single-cell lymphoblast growth more efficiently than do TK6 LCLs. It may also be desirable to use cells that do not have to be irradiated prior to use as a feeder layer. An $HPRT^-$ fibroblast cell strain could be used in this capacity if it were seeded into the wells of flat-bottomed 96-well plates several days prior to use. These cells would grow to confluence, enter G_0 and continue to condition the medium without interfering with the growth of the lymphoblast colonies (Bill Sugden, unpublished). Finally, the use of commercially prepared conditioned media supplements may help to improve the cloning efficiency of the LCLs used. These supplements (such as Human T-stim (Collaborative Biomedicals) or Hybri-Max conditioned media (Sigma)) may provide the same effect as the use of feeder cells without actually using feeder cells.

Given the variability exhibited between control LCLs

in these studies, it will be important to include more controls in future studies. In addition, if possible it would be desirable to include more RS+ and RS- LCLs. Unfortunately, only the four used in these studies are available at present. It may also be interesting to include FA LCLs from all of the defined complementation groups. In the present study, only HSC-99 was included as it had previously been shown to exhibit hypomutability after PUVA treatment. However, if the relationship between RS+ and FA cells is to be investigated further in this manner, FA LCLs from all of the complementation groups should be included.

The mutagen used in future studies should be one to which RS+ cells have been shown to be hypersensitive, specifically a crosslinking agent such as MMC or PUVA. This would permit the investigation of the relationship between mutagen hypersensitivity and mutability. In the present study, EMS was chosen as the mutagen because one of the objectives was to generate a universal hybridizing RS+ LCL. It was thought that this would be ensured by the use of a potent mutagen such as EMS. The use of a crosslinking agent to which both RS+ fibroblasts and lymphoblasts have been shown to be hypersensitive may provide additional insight into the defect in these cells. This is especially important given the experience in these studies with HSC-99. This LCL has previously been shown to be hypomutability by a DNA crosslinking agent to which it is also hypersensitive

(Papadopoulo et al., 1990a). However, in the present study, it was neither hypersensitive nor hypomutable by EMS. This demonstrates the importance of the mutagen used on the outcome.

4.2.3 Complementation studies between RS+ and Fanconi anaemia

As genetic heterogeneity has already been demonstrated in FA (Duckworth-Rysieki et al., 1985; Strathdee and Buchwald, 1991; Strathdee et al., 1992a), it was desirable to determine if any genetic relationship exists between any of the four complementation groups identified in FA and RS+ cells.

The results obtained in the hybridization experiments between the female RS+ LCL, LB-1-hygro, and LCLs from the four known FA complementation groups suggested a complex genetic relationship between the two conditions. The HCLs from fusion 7, between LB-1-hygro and HSC-230N, the FA LCL from complementation group B, were completely corrected with respect to all three parameters examined. The MMC sensitivity of these HCLs was not significantly different from that of the control. Similarly, both of the cytogenetic variables tested, DEB-induced chromosome aberrations and the RS effect, were found to be corrected to the level of the control LCL. Thus, complementation does appear to be occurring between these two LCLs indicating

that they belong to different complementation groups and have defects in different genes.

The HCLs from fusion 9 between LB-1-hygro and HSC-62N, the FA LCL from complementation group D, were corrected to the level of the control LCL with respect to both of the cytogenetic parameters tested. However, it is important to note here that although HSC-62N was found to be significantly more sensitive than HSC-3TO with respect to both the number of chromosome aberrations per cell and the percentage of cells with chromosome aberrations, it was not found to be significantly different from LB-1-hygro with respect to the number of aberrations per cell. This an interesting finding since the DEB-stress test has been reported to give "absolute discrimination" between FA patients and non-FA patients with respect to breaks per cell (Auerbach et al., 1989; Auerbach, 1993). If this is true, these findings indicate that LB-1-hygro might be considered positive for the DEB-stress test. In addition, HSC-62N was not significantly different from LB1x62N HCL 1 with respect to the number of aberrations per cell. However, HSC-62N was significantly different from both LB-1-hygro and LB1x62N HCL 1 with respect to the percentage of cells with chromosome aberrations.

Neither of the HCLs from fusion 9 were found to have complete correction of the MMC hypersensitivity but rather to be corrected only to the level of the RS+ parent. Thus,

only partial complementation appears to be occurring in these hybrids with respect to cellular MMC sensitivity. Complete complementation of the RS effect was observed but the degree of complementation of the hyperinduction of chromosome aberrations was difficult to establish given the relatively low number of aberrations observed in HSC-62N.

Full complementation of the RS effect was observed in the HCLs from fusion 8 between LB-1-*hygro* and HSC-536N, the FA LCL from complementation group C. However, only partial correction of the DEB-induced chromosome aberrations were observed. Both HCLs exhibited significantly higher levels of chromosome aberrations and a significantly higher proportion of cells exhibiting chromosome aberrations than the control LCL. Neither HCL was significantly different from LB-1-*hygro* with respect to these comparisons nor were they significantly different from HSC-62N. Thus, a partial complementation of the DEB-induced chromosome aberrations appears to have occurred but these HCLs are still exhibiting a response to the DEB-stress test that is within the range for FA. With respect to MMC sensitivities, all three HCLs tested were significantly more sensitive than the RS+ LCL thus indicating a lack of complementation of this defect. This indicates that LB-1-*hygro* and HSC-536N may share some genetic properties at least with respect to MMC cellular sensitivity and possibly with respect to DEB chromosomal sensitivity.

The HCLs from fusion 6 between LB-1-*hygro* and HSC-72N, the FA LCL from complementation group A, gave the most equivocal results. All five of the HCLs tested were found to be corrected with respect to the RS effect. With respect to the DEB-induced chromosome aberrations, two of the three hybrids tested were found to be corrected to the control level. The third HCL, LB1x72N HCL 5 was found to have both a significantly higher percentage of cells with chromosome aberrations and a significantly higher number of chromosome aberrations per cell than the control LCL. Similarly, this HCL was significantly more affected with respect to chromosome aberrations than was LB-1-*hygro*. Equivocal results were also obtained with the MMC dose response experiments. One of the HCLs, LB1x72N HCL 5 was corrected to the level of the control LCL while two other HCLs, LB1x72N HCLs 2 and 3, were corrected only to the level of LB-1-*hygro*. LB1x72N HCL 1 was corrected to a level that was intermediate between those of the RS+ and control LCLs. Finally, LB1x72N HCL 4 had a sensitivity that was intermediate between those of the two parental LCLs. The lack of correction of MMC sensitivity in LB1x72N HCL 4 may be due to the fact that this HCL is hypotetraploid (modal chromosome number = 87) and thus, appears to have lost some chromosomes in the course of establishing the cell line. It is possible, therefore, that most of the cells in the HCL have lost a chromosome or chromosomes which contain genes

critical for surviving MMC treatment. Unfortunately, given the relatively unstable nature of the chromosome complement of LCLs, it was not possible to establish with any certainty which chromosomes were lost.

It is interesting that LB1x72N HCL 4 was not abnormally sensitive to DEB-induced chromosome aberrations thus suggesting that different genes are involved in the repair of chromosome aberrations than are involved in the resistance to cell killing by MMC. A similar uncoupling of cell survival to MMC treatment and sensitivity to DEB-induced chromosome aberrations is seen with LB1x72N HCL 5 but in this case, normal MMC sensitivity is accompanied by a significantly higher than normal level of chromosome aberrations. It is more difficult to reconcile these observations with a loss of critical chromosomes since this hybrid does have a hypertetraploid modal chromosome number (106). It is impossible to be certain without karyotyping many cells, however, that all the chromosomes from both parental LCLs are represented in the majority of the cells. Phenotypic dissociation of chromosomal and cellular hypersensitivities has previously been reported in heterologous DNA transfer studies in FA fibroblasts (Diatloff-Zito et al., 1990; 1991; Moustacchi et al., 1991). It was suggested that the protein responsible for correcting these defects may contain two domains (Moustacchi et al., 1991). However, the results obtained here suggest that

different genes located on different chromosomes may be responsible for the chromosomal and cellular hypersensitivities of FA cells. The remaining observations of the HCLs in this fusion are consistent with a full complementation of the cytogenetic variables in the hybrids and a partial complementation of the MMC sensitivity.

Before addressing the possibility of a genetic relationship between FA and RS+ patients, it is important to first examine models of inheritance for FA alone. Family studies have indicated that FA is inherited in an autosomal recessive manner (Schroeder et al., 1976; McKusick, 1990, 227650). It is possible that FA is inherited in a simple, single gene autosomal recessive manner with the four different complementation groups each representing an enzyme involved in the same biochemical pathway. In this scenario, the products of the four genes would perform closely related functions, perhaps as subunits of a multi-enzyme complex or as enzymes for successive steps in a DNA repair pathway. However, a number of aspects of FA suggest that a more complex mode of inheritance may be involved. First, the presence of at least four complementation groups for a syndrome in which the major defect appears to be the repair of DNA crosslinks, a two-step process (Fujiwara et al., 1977; Fujiwara, 1982), seems excessive. Second, different researchers have found deficiencies in different enzymes for the same FA complementation group. For example, cells from

complementation group A have been shown to have an abnormal nuclease (Sakaguchi et al., 1991; 1992), decreased activity of a chromatin-associated endonuclease (Lambert et al., 1992) and decreased interleukin-6 (IL-6) activity (Rosselli et al., 1992). Similarly, decreased activity of another chromatin-associated endonuclease and of IL-6 have been reported in cells from complementation group D (Lambert et al., 1992; Rosselli et al., 1992). An abnormal intercellular distribution of topoisomerase activity has also been reported for FA cells although complementation group has not been determined in this case (Wunder et al., 1981). Finally, there have been reports of relatives of FA patients exhibiting FA-like hematological symptoms or malformations without appearing to have FA, including first degree relatives such as parents, siblings and offspring as well as more distant relatives such as grandparents, aunts and uncles and others (Gmyrek and Syllm-Rapoport, 1964 cited in Fanconi, 1967; Petridou and Barrett, 1990). While some of these relatives may actually represent undiagnosed cases of FA, others, especially more distantly related individuals, may be manifesting symptoms as the result of a carrier state. All of these observations concerning FA (the apparently excessive number of complementation groups, the evidence of multiple biochemical defects in single complementation groups and phenotypic expression in possible carriers) support a model of co-recessive inheritance (C-RI)

(Lambert and Lambert, 1989; 1992). In addition, the observations, both here and elsewhere, of dissociation of the cellular and chromosomal sensitivities to DNA crosslinking agents are also suggestive of more than one locus being involved in FA (Diatloff-Zito et al., 1990; 1991; Moustacchi et al., 1991). At this point, it is not possible to determine how many loci may be involved in the FA C-RI system or how many of those loci must be homozygous recessive for FA to be manifested as all of the complementation groups in FA may not have been identified. However, based on at least four complementation groups, a few simple models are possible. In the following equation, $C_{n,N}$ is the number of ways that an individual can be homozygous for defective alleles at n loci out of a defined set of N loci (Lambert and Lambert, 1992):

$$C_{n,N} = \frac{N!}{n!(N - n)!}$$

If there are three (N) loci in the C-RI system and an individual must be homozygous at two (n) of those loci to manifest FA, only three complementation groups are possible. Thus, at least four (N) loci must be involved in the FA C-RI system. If, as before, an individual must be homozygous at two (n) of those loci, six complementation groups are possible. If homozygosity is necessary at three of the four loci, four complementation groups are possible. Obviously, as the number of required loci increases, the incidence of the condition decreases or the carrier frequency for each

defective allele increases. For example, given an incidence of approximately 1 in 40,000 (Auerbach, 1992), when n = 2, approximately 15% of the population would be carriers for one of the defective alleles. When n = 3, approximately 50% of the population carries one of the defective alleles (Lambert and Lambert, 1992).

The C-RI model that will be discussed here is N = 4 and n = 2 since this is the simplest model that predicts at least four complementation groups for FA, the number that has been identified to date. However, other models are possible for this condition. Figure 6 outlines the model. Four loci, A, B, C and D, are involved in the system. Upper case letters represent the dominant normal allele and lower case letters represent the recessive defective allele(s). Because homozygosity is required at two of these loci, the at-risk genotypes are: aabbC-D-, aaB-ccD-, aaB-C-dd, A-bbccD-, A-bbC-dd and A-B-ccdd. The defective allele at a given locus for each FA complementation group need not be identical to that at the same locus in another complementation group but could be allelic mutations. Indeed, it may be this allelism that causes some of the differences seen in these studies between complementation groups with respect to cellular and chromosomal sensitivities to crosslinking agents. Assuming that both parents are heterozygous at all of the loci that the affected individual is homozygous for, the recurrence risk

Figure 6: A model of co-recessive inheritance (C-RI) for Fanconi anaemia (FA) and Roberts syndrome (RS+) patients. A. The four loci postulated to comprise the FA C-RI system. B. The six genotypes that would produce a phenotype of FA presuming that homozygosity is required at two of the four loci in the FA C-RI system. C. Genotypes tentatively assigned to each of the four FA complementation groups identified to date for the purposes of this model. D. The genotype assigned to RS+ patients to show overlap between the FA and RS+ C-RI systems. Note that the RS+ patients also show homozygosity at an additional locus (E) not included in the FA C-RI system.

FA C-RI System

A ___ B ___ C ___ D ___

FA At-Risk Genotypes

aabbC-D- aaB-ccD- aaB-C-dd

A-bbccD- A-bbC-dd A-B-ccdd

Example of Possible Genotypes for Defined FA Complementation Groups

A: A-bbccD-
B: aabbC-D-
C: A-B-ccdd
D: aaB-ccD-

RS+ Genotype

A-B-C-ddee

for siblings in this model is $(0.5)^{2n}$ or $(0.5)^4 = 6.25\%$. This is certainly much lower than what is observed in family studies. However, if the parents are homozygous for defective alleles at one of the loci involved, the recurrence risk to siblings increases accordingly. Homozygosity at one of the four loci involved in the C-RI system could be postulated to be causing the occurrence of FA-like symptoms in relatives of FA patients. In addition, given that there are four loci involved and homozygosity is required at only two of those loci, a sibling may present with one of the other possible affected genotypes than the proband. The very high predicted carrier frequencies for alleles in C-RI systems make these possibilities even more plausible (Lambert and Lambert, 1992).

There is little doubt that RS+ patients do not contain the same single gene mutation as any of the FA complementation groups. The lack of any kind of hematologic disorders in RS+ patients precludes this possibility. In addition, limb deformities tend to be more extensive in RS than in FA and mental retardation is more common in RS than in FA. However, given the clinical similarities and the similarities in cellular sensitivities to DNA crosslinking agents, it is possible to postulate a genetic association between FA patients and RS+ patients. It has already been suggest that these are allelic syndromes (Hall, 1987). If this was the case, it would be expected that the RS effect

and the DEB-induced chromosome aberrations would be corrected in hybrids because these are not present in FA and RS+ cells, respectively. The cellular sensitivity to MMC would be expected to be corrected to the level of the RS+ parent as it is the less sensitive of the two mutants. This is what was observed in the LB1x62N HCLs and, to some extent, in the LB1x72N HCLs but if HSC-62N and HSC-72N belong to different complementation groups, they cannot both be allelic to the RS+ defect. No complementation would be expected between these two FA LCLs if this were true and such complementation has been demonstrated previously (Strathdee et al., 1992a).

Another model for the genetic relationship between RS+ and some FA genotypes is that they contain defects in separate genes that encode proteins with related functions. These may include comprising different subunits of the same multi-enzyme complex responsible for the repair of DNA crosslinks or catalysing subsequent steps in the same DNA repair pathway. This appears to be the case between RS+ patients and FA patients from complementation group B given that these cell lines completely complemented each other. This is also the most probable genetic relationship between RS+ patients and FA patients from complementation groups A and D but, in these cases, one would expect the respective genes to encode proteins that interact to perform critical functions in the process of DNA repair because hybrids from

fusions 6 and 9 were only partially corrected with respect to cellular sensitivity to MMC. However, the lack of complete complementation of two of the three variables examined in hybrids from fusion 8 makes this an unlikely model for the relationship between RS+ patients and FA patients from complementation group C.

The C-RI model of inheritance in FA could be expanded to include RS+ patients. For example, as indicated in Figure 6, if the genotype aabbC-D- represents FA complementation group B and A-bbccD-, A-B-ccdd and aaB-ccD- represent complementation groups A, C and D, respectively, the defective loci in RS+ cells could include locus D. Again, the defects at this locus in the two cell types would probably not be identical but rather allelic mutations. In this model, locus D would control cellular sensitivity to MMC (and other DNA crosslinking agents) with the RS+ allele being less susceptible than the FA C allele. The RS+ C-RI system would also include another locus or loci that would account for some of the clinical and cellular differences between RS and FA.

In summary, the observations in these studies suggest a genetic association between RS+ patients and FA patients from some of the complementation groups, especially complementation group C. The findings are not consistent with an identical single gene mutation nor do they suggest an allelic association. FA patients from complementation

groups A, B and D probably contain mutations in different genes from those affected in RS+ genes. Some observations in FA are indicative of a complex pattern of inheritance such as co-recessive inheritance and it is possible that an overlap exists between RS+ patients and some FA patients with respect to a C-RI system.

The genetic relationship of RS- patients to RS+ and FA patients remains unclear. It is unlikely that RS- patients represent un-diagnosed FA cases because cells from these patients do not exhibit abnormal MMC sensitivity (Burns and Tomkins, 1989; present study). Thus, it is possible that RS- patients represent a distinct genetic entity. Baller-Gerold syndrome is one candidate for that genetic entity. Mis-diagnoses of RS+ and FA patients as Baller-Gerold syndrome indicate the clinical overlap between these conditions. It is possible that Baller-Gerold patients that are not subsequently re-diagnosed as RS+ or FA and RS- patients may contain the same genetic defect. Given the absence of any recognized cellular defects in either condition, it would not be possible to test this hypothesis by somatic cell hybridization. Only examination of the defect(s) at the gene level could prove this hypothesis.

The most likely FA complementation group to share a genetic relationship with RS+ patients is group C. A gene that corrects the cellular sensitivity of HSC-536 to mitomycin C and diepoxybutane has recently been isolated

(Strathdee *et al.*, 1992b). Efforts are presently underway to introduce the *FACC* gene into RS+ cells to determine if it is capable of correcting the cellular sensitivities in those cells. This gene has previously been shown to be incapable of correcting the defects in cells from FA complementation groups A, B and D (Strathdee *et al.*, 1992b). If correction of the cellular sensitivity to MMC is observed in RS+ cells transfected with the *FACC* gene, the RS+ copy of the gene will be sequenced to determine if any mutations are present.

If the *FACC* gene is incapable of correcting the cellular MMC sensitivity in RS+ cells, other FA genes should be assessed as they are cloned for their ability to correct the RS+ cells. In addition, if additional FA complementation groups are identified, complementation studies should be carried out between RS+ cells and FA cells from those complementation groups. Finally, if a gene is cloned that corrects the cellular sensitivity in RS+ cells, FA complementation groups for which a gene has not been identified should be assessed to determine if they possess mutations in the *RBS* gene.

Further evidence of a C-RI system of inheritance for FA could be provided by assessing the level of chromosome aberrations in hybrids between LCLs from different complementation groups. Hybrids were assessed only for correction of the cellular sensitivity to MMC and DEB exhibited by the parental LCLs (Strathdee *et al.*, 1992a).

No report of the level of chromosome aberrations in response to the DEB stress test has been made. Given the evidence of dissociation of the cellular and chromosomal sensitivities reported previously (Diatloff-Zito et al., 1990; 1991; Moustacchi et al., 1991) and observed in these studies, this question should definitely be addressed. A lack of complementation of chromosomal sensitivity to DEB would support the hypothesis of co-recessive inheritance in FA.

5. Conclusions

Complementation studies between RS+ LCLs and control, RS+ and RS- LCLs have provided a further characterization of these cells with respect to its genetic basis. Correction of both the RS effect and cellular MMC hypersensitivity in hybrids between RS+ and control LCLs strengthens the possibility of an association between these two phenomena in RS+ cells and that they arise because of a common defect in the Roberts syndrome (RBS) gene.

The high level of clinical heterogeneity in RS taken together with the presence of two subgroups of RS patients, those with the heterochromatin abnormality and those without it, is suggestive of a corresponding genetic heterogeneity. A lack of complementation in hybrids between RS+ LCLs from patients with diverse ethnic backgrounds suggests a low level of genetic heterogeneity between RS+ patients. Complete complementation of both RS+ cellular phenomena in hybrids between an RS+ LCL and two different RS- LCLs suggests that these conditions arise from different mutations either in the same gene (allelic heterogeneity) or in different genes (genetic heterogeneity).

The clinical and cellular similarities between RS and FA suggests the possibility of a genetic association

between these two disorders. Mutability studies of control, RS+, RS- and one FA A LCLs by EMS have indicated that RS+ LCLs may be hypomutable relative to control LCLs while RS- LCLs and the FA A LCL are not. Interpretation of these results is limited due to extreme variability in the data and a confounding effect of very low cloning efficiencies on the calculation of mutant frequency. Improvements to the experimental system may provide more definitive results.

Complete complementation of the RS effect, cellular MMC hypersensitivity and FA-specific chromosomal DEB hypersensitivity in hybrids between RS+ and FA B LCLs indicates that these LCLs belong to different complementation groups. Complete complementation of the RS effect and chromosomal DEB hypersensitivity and partial complementation of cellular MMC hypersensitivity in RS+ x FA A and RS+ x FA D hybrids is also suggestive of genetic heterogeneity between these LCLs. However, incomplete complementation of both chromosomal DEB hypersensitivity and cellular MMC hypersensitivity in hybrids between RS+ and FA C LCLs suggests a genetic association between these two LCLs. The basis of this association is unclear but is hypothesized to be an overlap between co-recessive inheritance systems for FA and RS.

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APPENDIX I:
Data from somatic cell hybridization experiments

Table AI-1: Chi-square Analyses of RS Effect Data for Fusions 1 and 2

Cell Lines Compared	X^2 Value[$]	P Value
HSC-3TO vs RS+[*]	50.00	<0.0001
HSC-3TO vs pooled RS+[**]	75.00	<0.0001
RS+ vs RS+x3TO Hybrid[***]	75.00	<0.0001
pooled RS+ vs pooled LB1x3TO[****]	175.00	<0.0001
pooled RS+ vs pooled R20x3TO[*****]	125.00	<0.0001

Note: No comparisons were done between HSC-3TO and hybrids because frequencies were 0/25 and 0/50, respectively (percentage affected cells was identical - 0%)

[$] df = 1
[*] Frequencies for LB-1 and R20 both 25/25, thus X^2 values are identical
[**] Pooled frequencies from both RS+ LCLs used here
[***] Frequencies for all hybrids are 0/50, thus all X^2 values are identical
[****] Pooled frequencies for three LB1x3TO hybrids
[*****] Pooled frequencies for two R20x3TO hybrids

Figure AI-1: Mitomycin C dose response curves for fusions 1 and 2. A. Responses of the control LCL, HSC-3TO (●) and the two RS+ LCLs, LB-1 (▽) and R20 (▼).

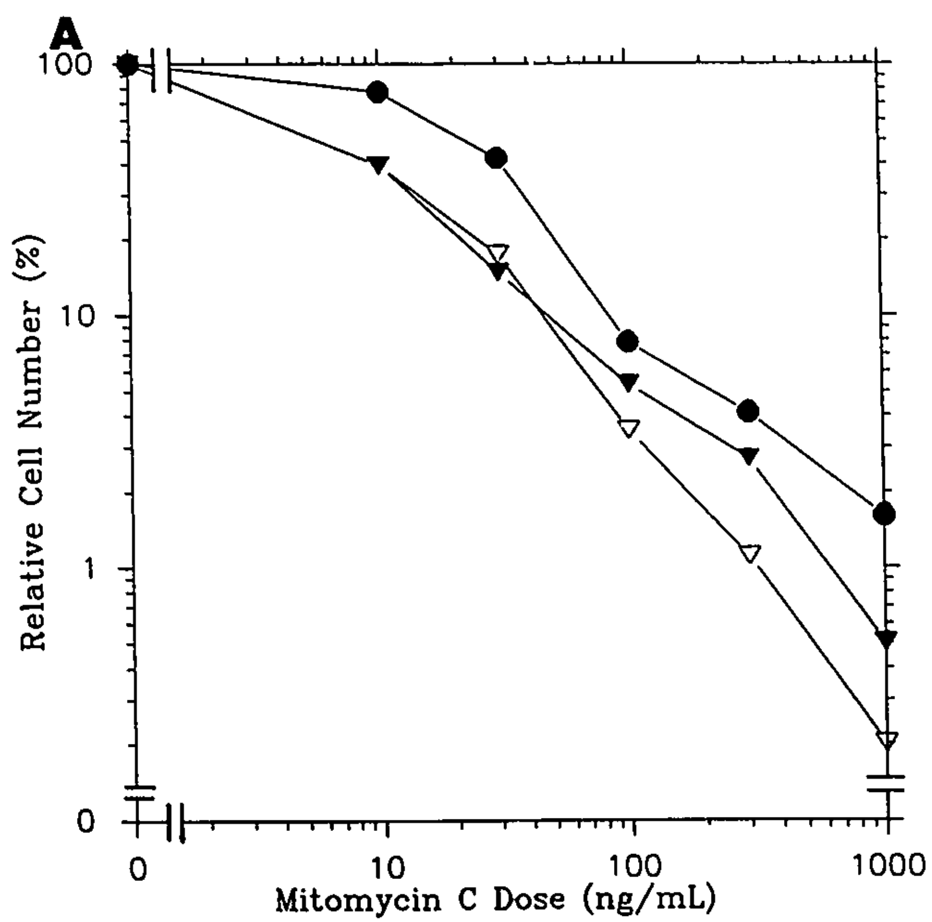
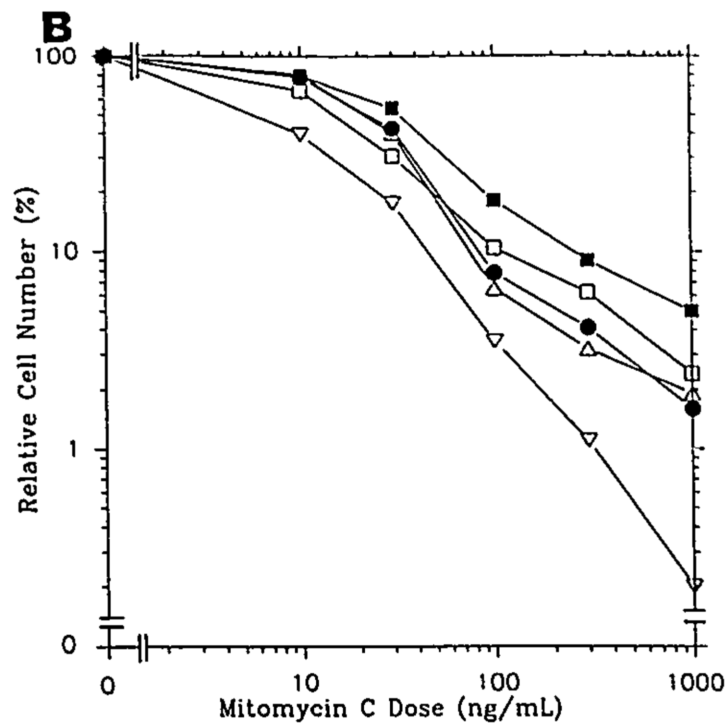

A
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

Figure AI-1 (cont'd): Mitomycin C dose response curves for fusions 1 and 2. B. Responses of the cell lines from fusion 1. HSC-3TO (●), LB-1 (▽), LB1x3TO HCL 1 (□), LB1x3TO HCL 2 (■) and LB1x3TO HCL 3 (△); C. Responses of the cell lines from fusion 2. HSC-3TO (●), R20 (▼), R20x3TO HCL 1 (□) and R20x3TO HCL 2 (■).


B
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

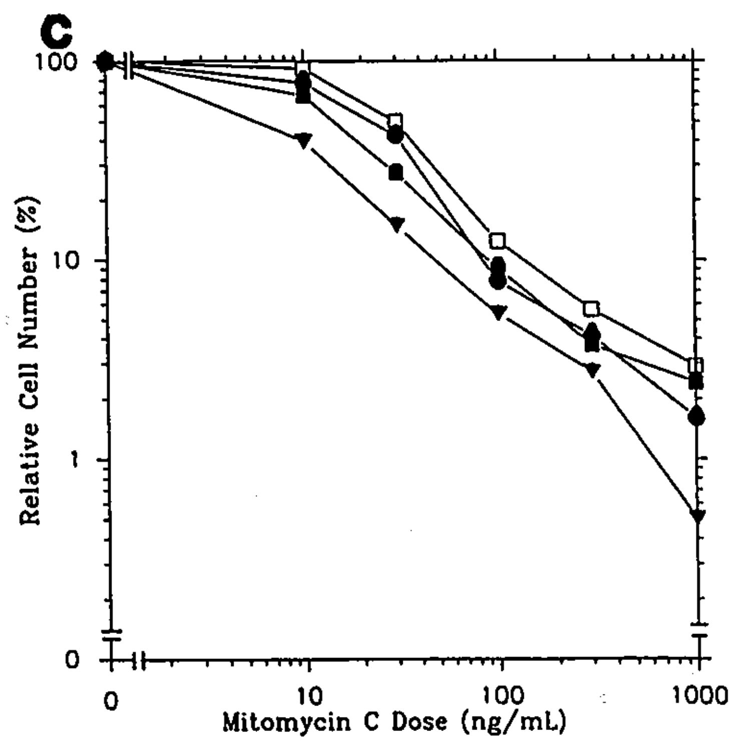

C
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

Table AI-2: Results of Mitomycin C Dose Response Experiments - Fusions 1 and 2

MMC Dose (ng/mL)	Relative Cell Number (%) (sd)							
	HSC-3TO	LB-1	R20	LB1x3TO HCL 1	LB1x3TO HCL 2	LB1x3TO HCL 3	R20x3TO HCL 1	R20x3TO HCL 2
0	100	100	100	100	100	100	100	100
10	78.0 (15.7)	39.7 (9.4)	39.7 (9.8)	91.9 (22.9)	67.4 (24.8)	66.4 (16.9)	79.7 (17.9)	81.0 (36.2)
30	42.5 (16.8)	17.4 (7.0)	14.8 (4.7)	49.9 (16.8)	27.3 (24.8)	30.6 (16.9)	54.1 (17.9)	40.3 (17.6)
100	7.8 (3.4)	3.5 (2.2)	5.3 (3.2)	12.4 (3.1)	9.0 (2.5)	10.4 (7.1)	18.1 (3.9)	6.5 (3.1)
300	4.1 (1.8)	1.1 (1.0)	2.7 (1.8)	5.6 (1.6)	3.7 (1.6)	6.2 (4.9)	9.0 (3.1)	3.2 (1.5)
1000	1.6 (1.0)	0.2 (0.2)	0.5 (0.4)	2.9 (1.5)	2.4 (0.8)	2.4 (1.6)	5.0 (1.4)	1.9 (1.5)

Table AI-3: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1, R20 and RS+ x Control Hybrids

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	7	329710.0	47102.0	20.56	<0.0001
Experiment	1	8733.6	8733.6	3.81	0.0563
Replicate	3	3038.0	1012.7	0.44	0.7240
Error	52	119140.0	2291.1		
Total	63	460620.0			
Grand Mean	1	1002700.0			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

HCL 2-1	HCL 1-1	HCL 1-3	HSC-3TO	HCL 1-2	HCL 2-2	R20	LB-1
280.8	168.0	149.8	115.0	103.7	95.71	45.7	42.6
	—	—	—	—	—		
			—	—	—	—	—

* Wholly Significant Difference
** Groups which are not significantly different from each other are indicated by lines (α = 0.05)

HCL 1-1 = LB-1x3TO HCL 1
HCL 1-2 = LB-1x3TO HCL 2
HCL 1-3 = LB-1x3TO HCL 3
HCL 2-1 = R20x3TO HCL 1
HCL 2-2 = R20x3TO HCL 2

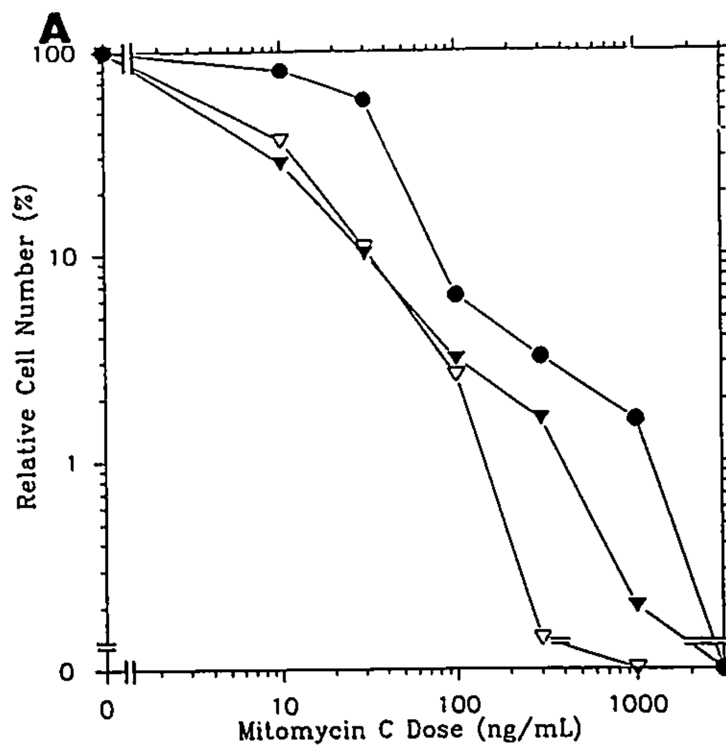
Table AI-4: Chi-square Analyses of RS Effect Data for Fusions 3, 4 and 5

Cell Lines Compared	X^2 Value[¶]	P Value
HSC-3TO vs LB-1	96.08	<0.0001
HSC-3TO vs R20	70.59	<0.0001
HSC-3TO vs pooled RS+[*]	116.99	<0.0001
HSC-3TO vs RS+xRS+[**]	75.00	<0.0001
HSC-3TO vs pooled R20xLB1[***]	100.00	<0.0001
LB-1 vs RS+xRS+	0.13[$]	0.7216
R20 vs RS+xRS+	0.00[$]	1.0000
pooled RS+ vs pooled R20xLB1	0.19[$]	0.6621
R20 vs RS-[****]	46.15	<0.0001
pooled RS+ vs pooled RS-[*****]	116.99	<0.0001
R20 vs RS+xRS- 1[#]	46.15	<0.0001
pooled RS+ vs pooled R20xLoGi[$]	116.99	<0.0001
R20 vs RS+xRS- 2[@]	42.32	<0.0001
pooled RS+ vs pooled R20xTiBu[&]	138.27	<0.0001
HSC-3TO vs RS+xRS- 2	0.13[$]	0.7216
HSC-3TO vs pooled R20xTiBu	0.04[$]	0.8376

Note: Comparisons were not made between cell lines when frequencies were identical (i.e. both 0/25).

¶ df = 1
$ Yates' correction for continuity was used in these cases
* Pooled frequency data from R20 and LB-1
** Frequency for both R20xLB-1 hybrids was 25/25, thus X^2 values are identical
*** Pooled frequency data for two R20xLB-1 hybrids
**** Frequency for both LoGi and TiBu was 0/25, thus X^2 values are identical
***** Pooled frequency data for LoGi and TiBu
\# Frequency for both R20xLoGi hybrids and 2/3 R20xTiBu hybrids was 0/25, thus X^2 values are identical
$ Pooled frequency data for two R20xLoGi hybrids
@ Frequency for 1/3 R20xTiBu hybrids was 1/25, thus X^2 value was different from that of other hybrids
& Pooled data for three R20xTiBu hybrids

Figure AI-2: Mitomycin C dose response curves for fusion 3. A. Responses of the control LCL, HSC-3TO (●) and the two RS+ LCLs, LB-1-hygro (▽) and R20-neo (▼); B. Responses of the control LCL, HSC-3TO (●), the two RS+ LCLs, LB-1-hygro (▽) and R20-neo (▼) and the two R20xLB1 HCLs, HCL 1 (□) and HCL 2 (■).


A
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

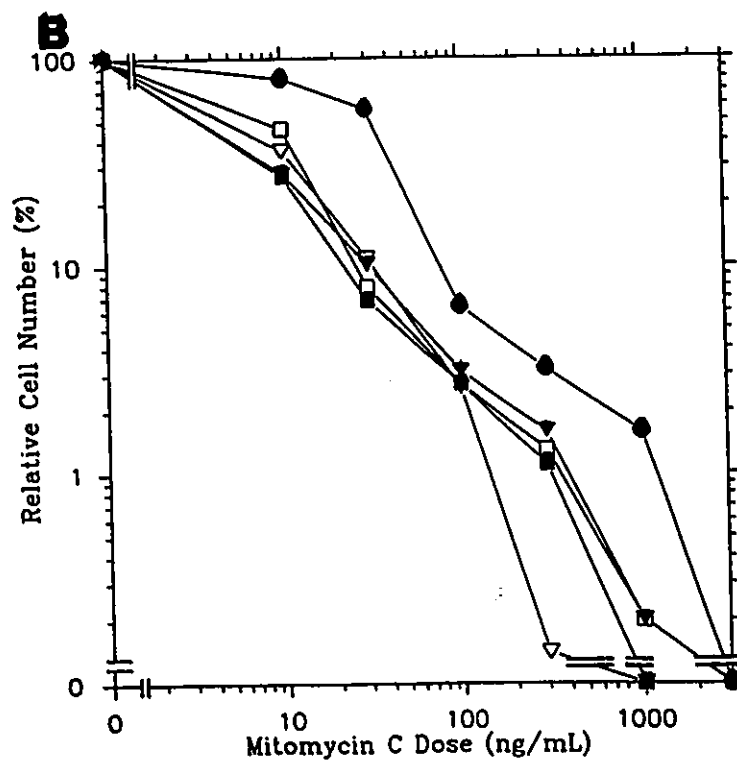

B
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

Table AI-5: Results of Mitomycin C Dose Response Experiments - Fusion 3

MMC Dose ng/mL	Relative Cell Number (%) (sd)				
	HSC-3TO	LB-1-*hygro*	R20-*neo*	R20xLB1 HCL 1	R20xLB1 HCL 2
0	100.0	100.0	100.0	100.0	100.0
10	80.2 (8.9)	36.0 (5.1)	27.8 (4.7)	46.0 (8.9)	26.9 (5.8)
30	57.1 (11.6)	10.8 (6.6)	10.1 (3.0)	7.9 (4.6)	6.8 (2.4)
100	6.4 (3.1)	2.6 (1.5)	3.1 (0.7)	2.7 (2.4)	2.7 (0.6)
300	3.2 (1.1)	0.1 (0.4)	1.6 (0.6)	1.3 (1.2)	1.1 (0.8)
1000	1.6 (0.7)	0.0 (0.0)	0.2 (0.3)	0.2 (0.4)	0.0 (0.1)
3000	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

Figure AI-3: Mitomycin C dose response curves for fusions 4 and 5. A. Responses of the control LCL, HSC-3TO (●), one RS+ LCL, R20-*neo* (▼) and the two RS- LCLs, TiBu-*hygro* (■) and LoGi-*hygro* (□).

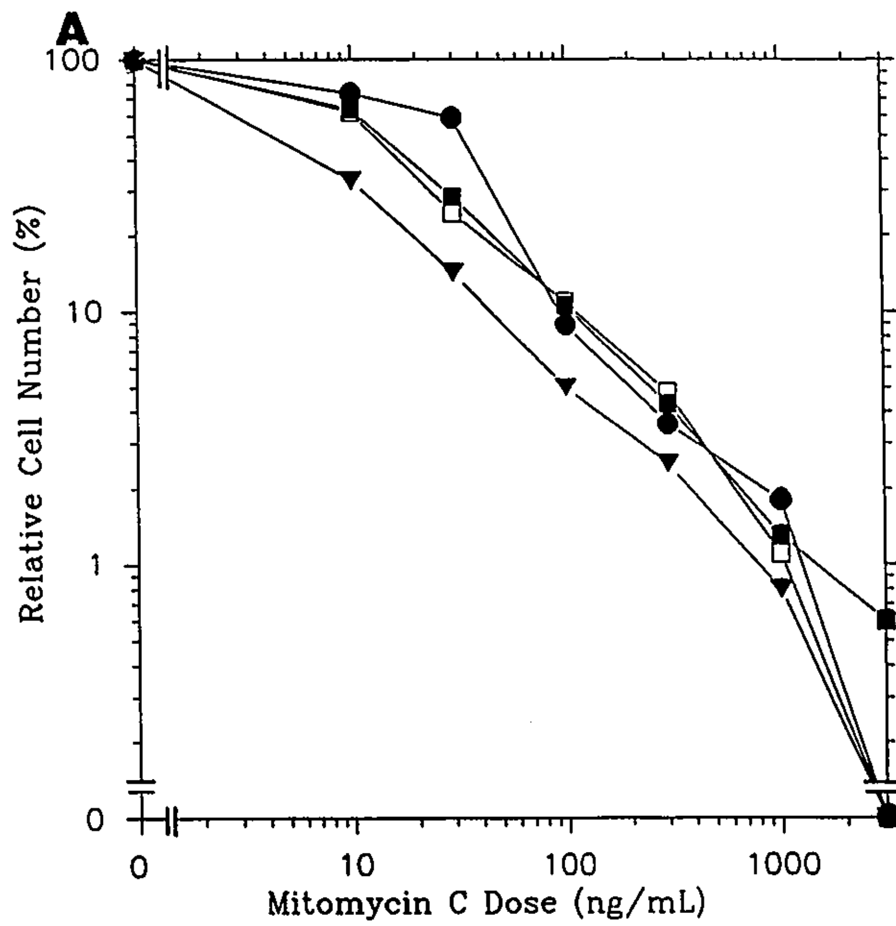
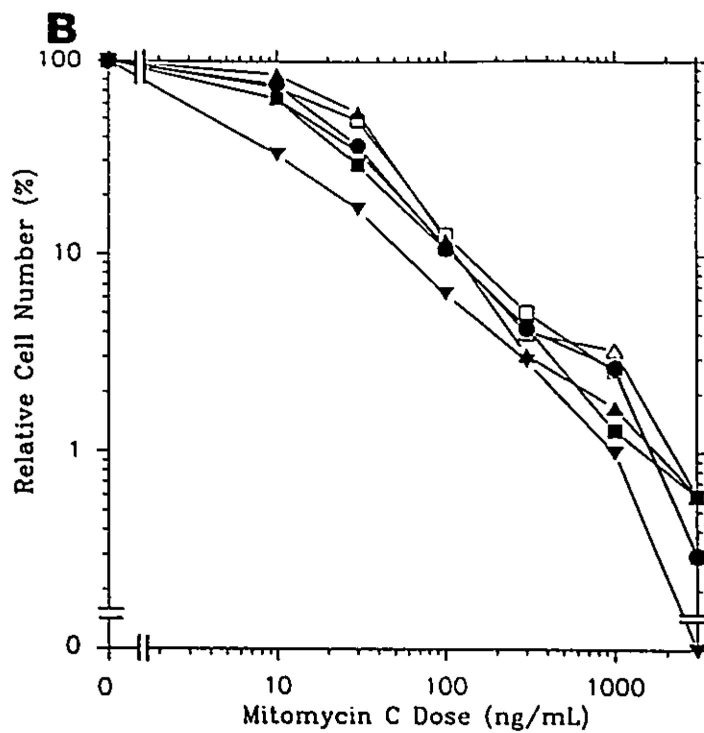
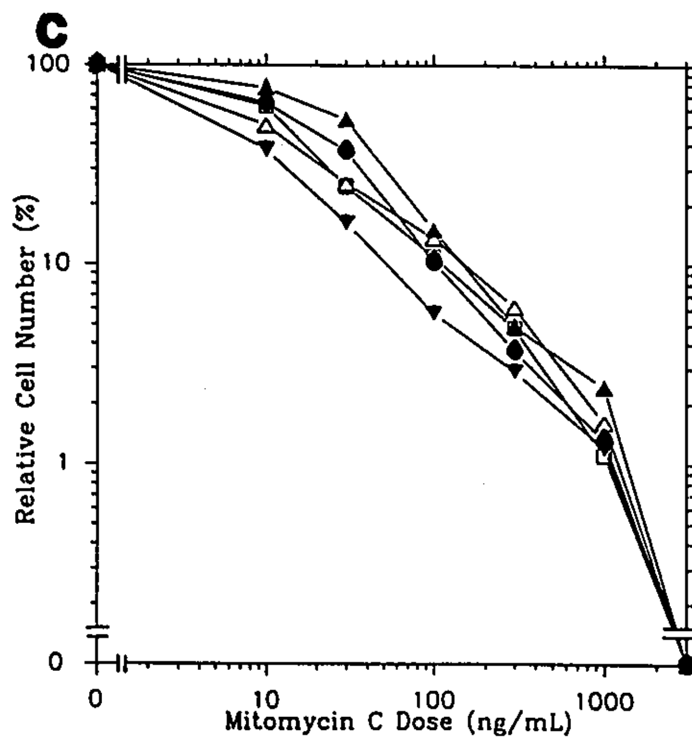

A
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

Figure AI-3 (cont'd): Mitomycin C dose response curves for fusions 4 and 5. B. Responses of the cell lines from fusion 4. HSC-3TO (●), R20-<u>neo</u> (▼), TiBu-<u>hygro</u> (■), R20xTiBu HCL 1 (▲), R20xTiBu HCL 2 (△) and R20xTiBu HCL 3 (□); C. Responses of the cell lines from fusion 5. HSC-3TO (●), R20-<u>neo</u> (▼), LoGi-<u>hygro</u> (□), R20xLoGi HCL 1 (▲) and R20xLoGi HCL 2 (△).



B
Relative Cell Number (%)
100
10
1
0
0
10
100
1000
Mitomycin C Dose (ng/mL)



C
Relative Cell Number (%)
100
10
1
0
0
10
100
1000
Mitomycin C Dose (ng/mL)

Table AI-6: Results of Mitomycin C Dose Response Experiments - Fusion 4

MMC Dose ng/mL	Relative Cell Number (%) (sd)					
	HSC-3TO	TiBu-*hygro*	R20-*neo*	R20xTiBu HCL 1	R20xTiBu HCL 2	R20xTiBu HCL 3
0	100.0	100.0	100.0	100.0	100.0	100.0
10	76.0 (11.0)	63.7 (9.9)	32.5 (3.7)	85.5 (10.6)	64.1 (18.9)	73.4 (11.0)
30	36.1 (10.8)	28.5 (6.5)	16.9 (2.8)	54.0 (10.9)	33.7 (4.5)	49.3 (17.8)
100	10.7 (0.9)	10.6 (2.4)	6.3 (1.5)	11.8 (2.6)	11.1 (2.9)	12.5 (2.3)
300	4.2 (0.9)	4.3 (1.0)	2.9 (0.6)	3.1 (1.1)	4.0 (1.5)	5.1 (1.8)
1000	2.7 (0.5)	1.3 (0.6)	1.0 (0.5)	1.7 (0.9)	3.3 (2.0)	2.6 (0.8)
3000	0.3 (0.1)	0.6 (0.3)	0.0 (0.0)	0.6 (0.3)	0.6 (0.3)	0.3 (0.5)

Table AI-7: Results of Mitomycin C Dose Response Experiments - Fusion 5

MMC Dose ng/mL	Relative Cell Number (%) (sd)				
	HSC-3TO	LoGi-hygro	R20-neo	R20xLoGi HCL 1	R20xLoGi HCL 2
0	100.0	100.0	100.0	100.0	100.0
10	64.5 (17.2)	62.1 (12.0)	37.6 (12.7)	49.7 (6.6)	76.5 (15.6)
30	37.2 (15.7)	24.6 (4.3)	16.1 (5.8)	25.4 (6.4)	53.3 (22.6)
100	10.3 (5.2)	10.9 (3.3)	5.7 (1.8)	13.4 (4.3)	14.8 (3.7)
300	3.7 (1.5)	4.8 (2.9)	2.9 (1.0)	6.1 (2.0)	4.9 (1.2)
1000	1.3 (0.6)	1.1 (1.3)	1.2 (0.8)	1.6 (1.0)	2.4 (1.6)
3000	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)

Table AI-8: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1-*hygro*, R20-*neo* and LB-1xR20 HCLs

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	4	64127.0	16032.0	51.03	<0.0001
Experiment	3	1999.7	666.58	2.12	0.1084
Replicate	3	590.8	196.98	0.63	0.6009
Error	53	16651.0	314.17		
Total	63	83368.0			
Grand Mean	1	160380.0			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

HSC-3TO	LB-1	R20	R20xLB-1 1	R20xLB-1 2
99.7	42.1	32.0	34.1	18.0

(Lines: LB-1 — R20 — R20xLB-1 1; R20 — R20xLB-1 1 — R20xLB-1 2)

* Wholly Significant Difference
** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-9: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, TiBu-<u>hygro</u>, R20-<u>neo</u> and R20xTiBu HCLs

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	5	40942.0	8188.4	14.69	<0.0001
Experiment	1	63.9	63.9	0.11	0.7367
Replicate	3	1341.7	447.2	0.80	0.5003
Error	38	21180.0	557.4		
Total	47	63528.0			
Grand Mean	1	562860.0			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

R20	TiBu	HSC-3TO	R20xTiBu 2	R20xTiBu 1	R20xTiBu 3
51.9	98.2	114.1	116.9	120.5	148.1
	————	————	————	————	
		————	————	————	————

* Wholly Significant Difference

** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-10: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LoGi-*hygro*, R20-*neo* and R20xLoGi HCLs

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	4	83346.0	20836.0	22.02	<0.0001
Experiment	3	27098.0	9032.8	9.54	<0.0001
Replicate	3	8067.3	2869.1	3.03	0.0380
Error	49	46373.0	946.4		
Total	59	16542.0			
Grand Mean	1	841950.0			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

R20	LoGi	HSC-3TO	R20xLoGi 1	R20xLoGi 2
52.1	102.0	96.4	118.8	152.6
	————	————	————	
			————	————

* Wholly Significant Difference

** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-11: Chi-square Analyses for RS Effect for Fusion 6

Cell Lines Compared	X^2 Value*	P Value
HSC-3TO x LB-1-*hygro*	96.08	<0.0001
LB-1-*hygro* x HSC-72N	96.08	<0.0001
LB-1-*hygro* x LB-1x72N**	70.67	<0.0001
LB-1-*hygro* x pooled LB-1x72N***	268.31	<0.0001

Note: Comparisons were not made between cell lines with identical frequencies (i.e. both 0/25).

* df = 1
** Frequency for all five LB-1x72N hybrids was 0/25, thus X^2 values were identical
*** Pooled frequency data for five hybrids

Table AI-12: Chi-square Analysis of Percentage of Cells with Chromosome Aberrations - Fusion 6

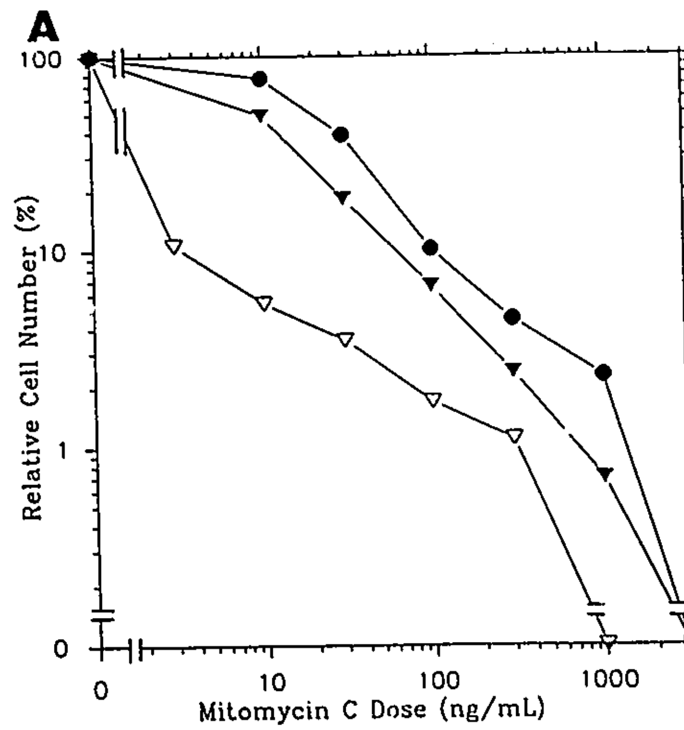
Cell Lines Tested	X^2 Value*	P Value
HSC-3TO & LB-1-hygro	2.198	0.1382
HSC-3TO & HSC-72N	162.065	<0.0001
HSC-3TO & LB1x72N HCL 1	2.198	0.1382
HSC-3TO & LB1x72N HCL 4	3.556	0.0593
HSC-3TO & LB1x72N HCL 5	14.881	0.0001
LB-1-hygro & HSC-72N	142.029	<0.0001
LB-1-hygro & LB1x72N HCL 1	0.000	1.0000
LB-1-hygro & LB1x72N HCL 4	0.177	0.6740
LB-1-hygro & LB1x72N HCL 5	6.368	0.0116
HSC-72N & LB1x72N HCL 1	142.029	<0.0001
HSC-72N & LB1x72N HCL 4	135.838	<0.0001
HSC-72N & LB1x72N HCL 5	102.984	<0.0001

* df = 1

Table AI-13: Two-sample t-test Results of Number of Chromosome Aberrations Per Cell - Fusion 6

Cell Lines Tested	t Value	df	P Value
HSC-3TO & LB-1-hygro	-1.20	79	0.2336
HSC-3TO & HSC-72N	-11.26	49	<0.0001
HSC-3TO & LB1x72N HCL 1	-1.18	67	0.2420
HSC-3TO & LB1x72N HCL 4	-1.33	86	0.1870
HSC-3TO & LB1x72N HCL 5	-3.24	55	0.0021
LB-1-hygro & HSC-72N	-10.97	51	<0.0001
LB-1-hygro & LB1x72N HCL 1	-0.21	90	0.8342
LB-1-hygro & LB1x72N HCL 4	0.00	96	1.0000
LB-1-hygro & LB1x72N HCL 5	-2.51	66	0.0146
HSC-72N & LB1x72N HCL 1	10.82	53	<0.0001
HSC-72N & LB1x72N HCL 4	11.00	50	<0.0001
HSC-72N & LB1x72N HCL 5	9.58	60	<0.0001

Figure AI-4: Mitomycin C dose response curves for cell lines from fusion 6. A. Responses of the control LCL, HSC-3TO (●), LB-1-<u>hygro</u> (▼) and the FA LCL from complementation group A, HSC-72N (▽); B. Responses of HSC-3TO (●), LB-1-<u>hygro</u> (▼), HSC-72N (▽), LB1x72N HCL 1 (□), LB1x72N HCL 2 (■), LB1x72N HCL 3 (△), LB1x72N HCL 4 (▲) and LB1x72N HCL 5 (○).


A
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

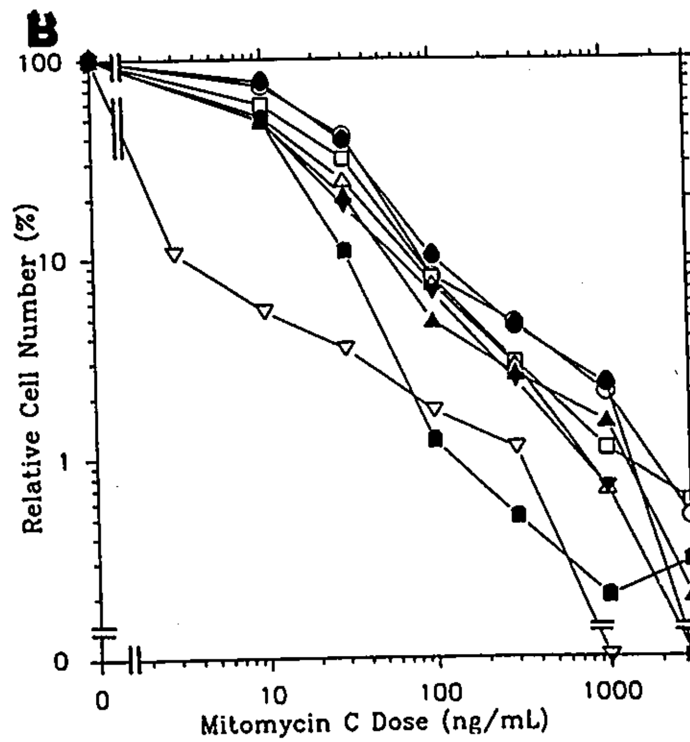

B
100
10
1
0
Relative Cell Number (%)
0
10
100
1000
Mitomycin C Dose (ng/mL)

Table AI-14: Results of Mitomycin C Dose Response Experiments - Fusion 6

MMC Dose ng/mL	Relative Cell Number (%) (sd)							
	HSC-3TO	HSC-72N	LB-1-hygro	LB1x72N HCL 1	LB1x72N HCL 2	LB1x72N HCL 3	LB1x72N HCL 4	LB1x72N HCL 5
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
3	-	10.6	-	-	-	-	-	-
	-	(5.1)	-	-	-	-	-	-
10	76.9	5.4	49.2	59.1	51.1	48.6	49.1	73.5
	(13.3)	(2.7)	(9.6)	(13.3)	(11.4)	(10.6)	(12.3)	(18.0)
30	39.0	3.5	18.4	31.6	24.8	20.7	10.8	41.2
	(13.8)	(1.6)	(3.7)	(14.0)	(9.4)	(5.6)	(3.3)	(24.5)
100	10.1	1.7	6.6	7.9	7.4	4.8	1.2	8.1
	(3.9)	(1.0)	(1.1)	(5.5)	(2.3)	(2.1)	(0.8)	(3.7)
300	4.5	1.1	2.4	3.0	2.9	2.6	0.5	4.7
	(2.3)	(1.3)	(1.1)	(1.9)	(2.3)	(1.3)	(0.5)	(1.8)
1000	2.3	0.0	0.7	1.1	0.7	1.5	0.2	2.1
	(1.1)	(0.1)	(0.5)	(1.1)	(0.7)	(1.5)	(0.3)	(1.1)
3000	0.1	-	0.0	0.6	0.0	0.2	0.3	0.5
	(0.2)	-	(0.0)	(1.0)	(0.0)	(0.4)	(0.8)	(0.4)

- = dose not used

Table AI-15: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1-*hygro*, HSC-72N and LB-1x72N HCLs (I)

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	4	139380.0	34845.00	52.45	<0.0001
Experiment	2	5862.2	2931.10	4.41	0.0189
Replicate	3	1640.4	546.79	0.82	0.4893
Error	38	252450.0	664.35		
Total	47	172130.0			
Grand Mean	1	243930.0			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

HSC-3TO	LB1x72N 1	LB-1	LB1x72N 4	HSC-72N
143.3	92.4	60.5	28.1	3.5

* Wholly Significant Difference

** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-16: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1-*hygro*, HSC-72N and LB-1x72N HCLs (II)

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	5	94612.00	18922.00	9.34	<0.0001
Experiment	1	31.26	31.26	0.02	0.9019
Replicate	3	410.85	136.95	0.07	0.9768
Error	34	68919.00	2027.00		
Total	43	163970.00			
Grand Mean	1	266990.00			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

HSC-3TO	LB1x72N 5	LB1x72N 2	LB1x72N 3	LB-1	HSC-72N
139.2	118.0	72.7	60.6	55.1	2.7

* Wholly Significant Difference
** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-17: Chi-square Analyses for RS Effect for Fusion 7

Cell Lines Compared	X^2 Value*	P Value
HSC-3TO x LB-1-*hygro*	96.08	<0.0001
LB-1-*hygro* x HSC-230N	96.08	<0.0001
LB-1-*hygro* x LB-1x230N**	70.67	<0.0001
LB-1-*hygro* x pooled LB-1x230N***	194.70	<0.0001

Note: Comparisons were not made between cell lines with identical frequencies (i.e. both 0/25).
* df = 1
** Frequency for all four LB-1x230N hybrids was 0/25, thus X^2 values were identical
*** Pooled frequency data for four hybrids

Table AI-18: Chi-square Analysis of Percentage of Cells with Chromosome Aberrations - Fusion 7

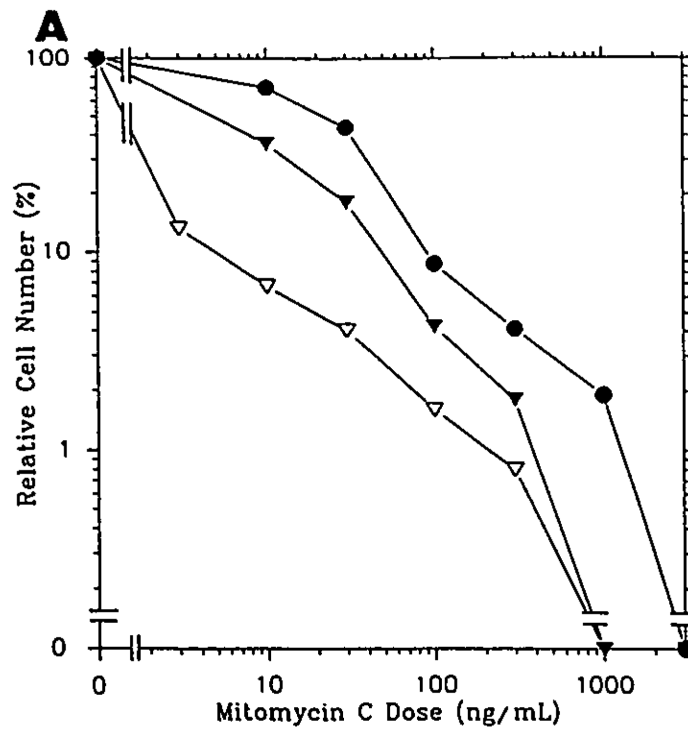
Cell Lines Tested	X^2 Value*	P Value
HSC-3TO & LB-1-*hygro*	2.198	0.1382
HSC-3TO & HSC-230N	169.065	<0.0001
HSC-3TO & LB1x230N HCL 2	0.307	0.5795
HSC-3TO & LB1x230N HCL 3	2.083	0.1489
LB-1-*hygro* & HSC-230N	149.414	<0.0001
LB-1-*hygro* & LB1x230N HCL 2	0.889	0.3457
LB-1-*hygro* & LB1x230N HCL 3	7.680	0.0056
HSC-230N & LB1x230N HCL 2	162.585	<0.0001
HSC-230N & LB1x230N HCL 3	184.320	<0.0001

* df = 1

Table AI-19: Two-sample t-test Results of Number of Chromosome Aberrations Per Cell - Fusion 7

Cell Lines Tested	t Value	df	P Value
HSC-3TO & LB-1-*hygro*	-1.20	79	0.2336
HSC-3TO & HSC-230N	-9.73	49	<0.0001
HSC-3TO & LB1x230N HCL 2	-0.39	96	0.6974
HSC-3TO & LB1x230N HCL 3	1.02	79	0.3108
LB-1-*hygro* & HSC-230N	-9.61	49	<0.0001
LB-1-*hygro* & LB1x230N HCL 2	0.87	86	0.3866
LB-1-*hygro* & LB1x230N HCL 3	1.98	60	0.0522
HSC-230N & LB1x230N HCL 2	9.70	49	<0.0001
HSC-230N & LB1x230N HCL 3	9.79	49	<0.0001

Figure AI-5: Mitomycin C dose response curves for cell lines from fusion 7. A. Responses of the control LCL, HSC-3TO (●), LB-1-*hygro* (▼) and the FA LCL from complementation group B, HSC-230N (▽); B. Responses of HSC-3TO (●), LB-1-*hygro* (▼), HSC-230N (▽), LB1x230N HCL 1 (□), LB1x230N HCL 2 (■), LB1x230N HCL 3 (△) and LB1x230N HCL 4 (▲).


A
Relative Cell Number (%)
100
10
1
0
0
10
100
1000
Mitomycin C Dose (ng/mL)

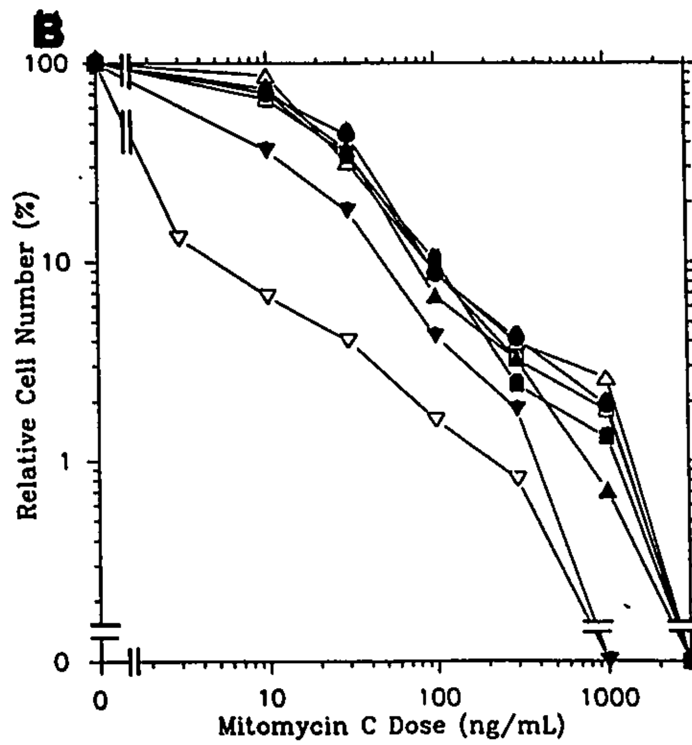

B
Relative Cell Number (%)
100
10
1
0
0
10
100
1000
Mitomycin C Dose (ng/mL)

Table AI-20: Results of Mitomycin C Dose Response Experiments - Fusion 7

MMC Dose ng/mL	Relative Cell Number (%) (sd)						
	HSC-3TO	HSC-230N	LB-1-hygro	LB1x230N HCL 1	LB1x230N HCL 2	LB1x230N HCL 3	LB1x230N HCL 4
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
3	- -	13.1 (3.9)	- -	- -	- -	- -	- -
10	70.6 (8.8)	6.7 (2.2)	36.1 (8.0)	66.3 (12.4)	71.2 (12.7)	86.6 (8.8)	74.3 (12.0)
30	43.6 (13.8)	4.0 (1.7)	17.9 (3.6)	35.3 (8.9)	33.8 (8.0)	31.5 (6.4)	36.6 (12.0)
100	8.7 (2.1)	1.6 (0.9)	4.2 (2.0)	8.9 (3.3)	10.1 (3.9)	8.8 (3.6)	6.7 (1.9)
300	4.1 (0.9)	0.8 (0.5)	1.8 (1.6)	3.2 (1.8)	2.4 (1.4)	3.9 (1.2)	3.2 (1.0)
1000	1.9 (0.8)	0.0 (0.0)	0.0 (0.0)	1.8 (0.9)	1.3 (1.3)	2.6 (1.5)	0.7 (0.9)
3000	0.0 (0.1)	- -	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

- = dose not used

Table AI-21: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1-*hygro*, HSC-230N and LB-1x230N HCLs (I)

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	3	55687.00	18562.00	32.91	<0.0001
Experiment	1	246.14	246.14	0.44	0.5152
Replicate	3	589.38	196.46	0.35	0.7907
Error	24	13537.00	564.04		
Total	31	70059.00			
Grand Mean	1	146980.00			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

HSC-3TO	LB1x230N 1	LB-1	HSC-230N
114.9	95.6	54.4	6.2

________________________ (line under HSC-3TO and LB1x230N 1)

* Wholly Significant Difference

** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-22: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1-*hygro*, HSC-230N and LB-1x230N HCLs (II)

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	5	83934.00	16787.00	22.03	<0.0001
Experiment	1	6767.30	6767.30	8.88	0.0053
Replicate	3	2697.80	882.59	1.16	0.3399
Error	34	25909.00	762.04		
Total	43	119260.00			
Grand Mean	1	286210.00			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

LB1x230N 3	HSC-3TO	LB1x230N 2	LB1x230N 4	LB-1	HSC-230N
124.1	109.8	99.9	84.4	41.0	4.1
________	________	________	________		
				________	________

* Wholly Significant Difference

** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-23: Chi-square Analyses for RS Effect for Fusion 8

Cell Lines Compared	X^2 Value*	P Value
HSC-3TO x LB-1-*hygro*	96.08	<0.001
LB-1-*hygro* x HSC-536N	96.08	<0.001
LB-1-*hygro* x LB-1x536N**	70.67	<0.001
LB-1-*hygro* x pooled LB-1x536N***	170.14	<0.001

Note: Comparisons were not made between cell lines with identical frequencies (i.e. both 0/25).

* df = 1
** Frequency for all three LB-1x536N hybrids was 0/25, thus X^2 values were identical
*** Pooled frequency data for three hybrids

Table AI-24: Chi-square Analysis of Percentage of Cells with Chromosome Aberrations - Fusion 8

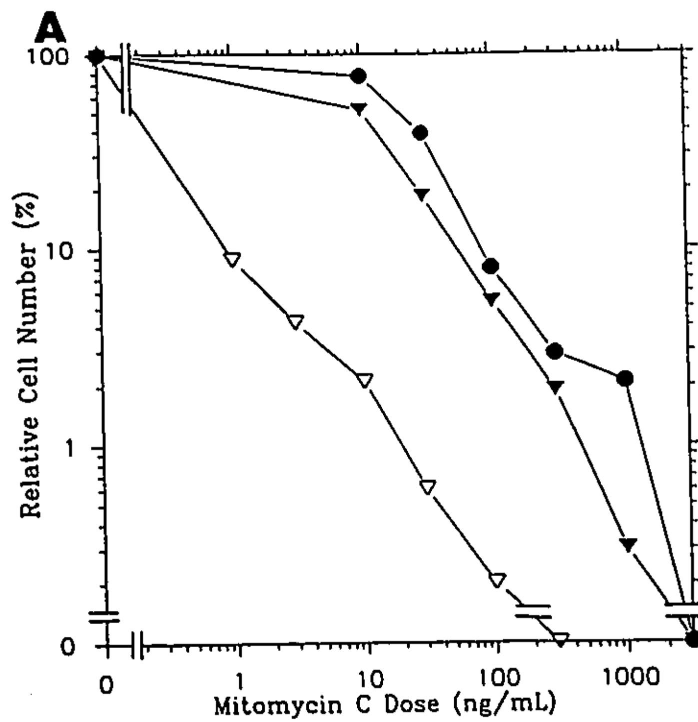
Cell Lines Tested	X^2 Value*	P Value
HSC-3TO & LB-1-*hygro*	2.198	0.1382
HSC-3TO & HSC-536N	162.065	<0.0001
HSC-3TO & LB1x536N HCL 1	8.665	0.0032
HSC-3TO & LB1x536N HCL 3	10.631	0.0011
LB-1-*hygro* & HSC-536N	142.029	<0.0001
LB-1-*hygro* & LB1x536N HCL 1	2.381	0.1228
LB-1-*hygro* & LB1x536N HCL 3	3.554	0.0598
HSC-536N & LB1x536N HCL 1	118.555	<0.0001
HSC-536N & LB1x536N HCL 3	113.187	<0.0001

* df = 1

Table AI-25: Two-sample t-test Results of Number of Chromosome Aberrations Per Cell - Fusion 8

Cell Lines Tested	t Value	df	P Value
HSC-3TO & LB-1-*hygro*	-1.20	79	0.2336
HSC-3TO & HSC-536N	-9.60	49	<0.0001
HSC-3TO & LB1x536N HCL 1	-2.11	79	0.0380
HSC-3TO & LB1x536N HCL 3	-2.27	60	0.0268
LB-1-*hygro* & HSC-536N	-9.38	50	<0.0001
LB-1-*hygro* & LB1x536N HCL 1	-0.74	97	0.4610
LB-1-*hygro* & LB1x536N HCL 3	-1.39	77	0.1684
HSC-536N & LB1x536N HCL 1	9.25	50	<0.0001
HSC-536N & LB1x536N HCL 3	8.89	53	<0.0001

Figure AI-6: Mitomycin C dose response curves for the cell lines from fusion 8. A. Responses of the control LCL, HSC-3TO (●), LB-1-*hygro* (▼) and the FA LCL from complementation group C, HSC-536N (▽); B. Responses of HSC-3TO (●), LB-1-*hygro* (▼), HSC-536N (▽), LB1x536N HCL 1 (□), LB1x536N HCL 2 (■) and LB1x536N HCL 3 (△).


A
Relative Cell Number (%)
100
10
1
0
0
1
10
100
1000
Mitomycin C Dose (ng/mL)

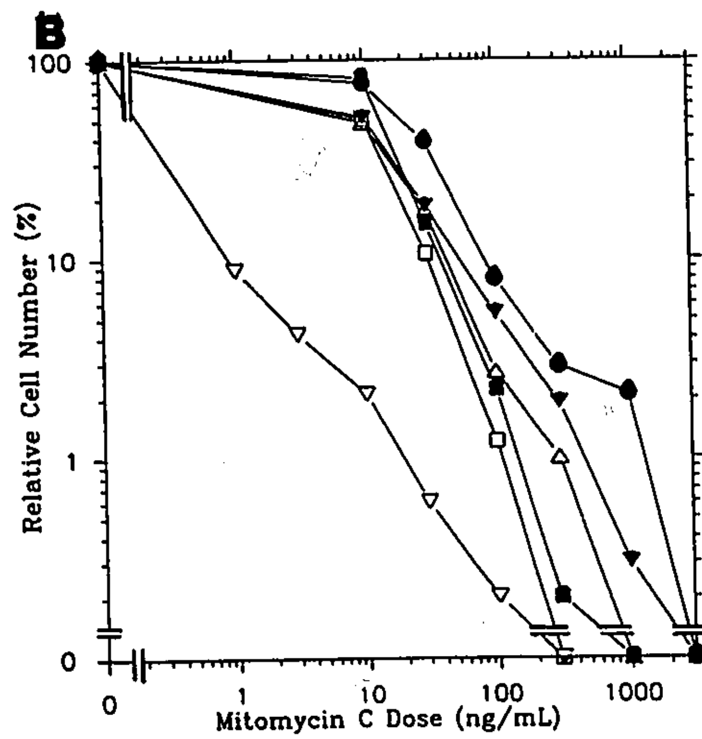

B
Relative Cell Number (%)
100
10
1
0
0
1
10
100
1000
Mitomycin C Dose (ng/mL)

Table AI-26: Results of Mitomycin C Dose Response Experiments - Fusion 8

MMC Dose ng/mL	Relative Cell Number (%) (sd)					
	HSC-3TO	HSC-536N	LB-1-*hygro*	LB1x536N HCL 1	LB1x536N HCL 2	LB1x536N HCL 3
0	100.0	100.0	100.0	100.0	100.0	100.0
1	-	8.8	-	-	-	-
	-	(1.0)	-	-	-	-
3	-	4.2	-	-	-	-
	-	(1.5)	-	-	-	-
10	76.9	2.1	51.3	50.5	81.1	48.2
	(12.1)	(0.6)	(9.6)	(23.2)	(22.3)	(12.8)
30	38.8	0.6	18.3	10.6	15.0	17.3
	(10.4)	(0.5)	(4.8)	(6.8)	(4.7)	(5.0)
100	7.9	0.2	5.3	1.2	2.2	2.7
	(1.3)	(0.3)	(1.4)	(0.9)	(1.0)	(1.3)
300	2.9	0.0	1.9	0.0	0.2	1.0
	(0.8)	(0.0)	(1.3)	(0.0)	(0.4)	(0.7)
1000	2.1	-	0.3	0.0	0.0	0.1
	(0.7)	-	(0.3)	(0.0)	(0.0)	(0.2)
3000	0.0	-	0.0	0.0	0.0	0.0
	(0.0)	-	(0.0)	(0.0)	(0.0)	(0.0)

- = dose not used

Table AI-27: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1-hygro, HSC-536N and LB-1x536N HCLs

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	5	73735.00	14747.00	131.48	<0.0001
Experiment	2	44.19	22.10	0.20	0.8218
Replicate	3	106.05	35.35	0.32	0.8143
Error	49	5496.00	112.16		
Total	59	79381.00			
Grand Mean	1	136880.00			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

HSC-3TO	LB-1	LB1x536N 3	LB1x536N 2	LB1x536N 1	HSC-536N
106.3	52.6	37.9	35.7	27.6	1.2

(A line is drawn under LB1x536N 3, LB1x536N 2 and LB1x536N 1.)

* Wholly Significant Difference
** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

Table AI-28: Chi-square Analyses for RS Effect for Fusion 9

Cell Lines Compared	X^2 Value*	P Value
HSC-3TO x LB-1-*hygro*	96.08	<0.0001
LB-1-*hygro* x HSC-62N	96.08	<0.0001
LB-1-*hygro* x LB-1x62N**	96.08	<0.0001
LB-1-*hygro* x pooled LB-1x62N***	145.55	<0.0001

Note: Comparisons were not made between cell lines with identical frequencies (i.e. both 0/25).

* df = 1
** Frequency for both LB-1x62N hybrids was 0/25, thus X^2 values were identical
*** Pooled frequency data for two hybrids

Table AI-29: Chi-square Analysis of Percentage of Cells with Chromosome Aberrations - Fusion 9

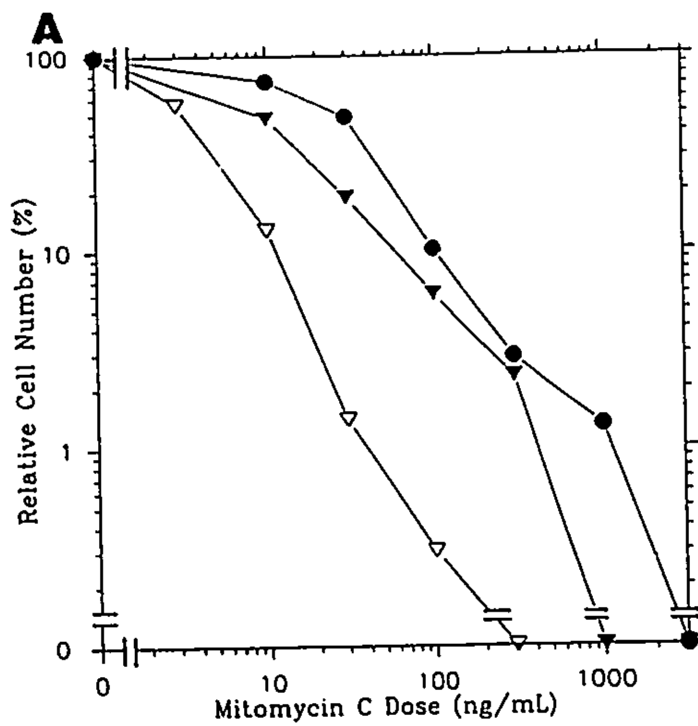
Cell Lines Tested	X^2 Value*	P Value
HSC-3TO & LB-1-*hygro*	2.198	0.1382
HSC-3TO & HSC-62N	14.881	<0.0001
HSC-3TO & LB1x62N HCL 1	2.198	0.1382
HSC-3TO & LB1x62N HCL 2	0.421	0.5164
LB-1-*hygro* & HSC-62N	6.368	0.0116
LB-1-*hygro* & LB1x62N HCL 1	0.000	1.0000
LB-1-*hygro* & LB1x62N HCL 2	4.348	0.0371
HSC-62N & LB1x62N HCL 1	6.368	0.0116
HSC-62N & LB1x62N HCL 2	18.980	<0.0001

* df = 1

Table AI-30: Two-sample t-test Results of Number of Chromosome Aberrations Per Cell - Fusion 9

Cell Lines Tested	t Value	df	P Value
HSC-3TO & LB-1-hygro	-1.20	79	0.2336
HSC-3TO & HSC-62N	-2.18	55	0.0336
HSC-3TO & LB1x62N HCL 1	-1.04	89	0.3012
HSC-3TO & LB1x62N HCL 2	0.45	94	0.6538
LB-1-hygro & HSC-62N	-1.52	66	0.1332
LB-1-hygro & LB1x62N HCL 1	0.27	94	0.7878
LB-1-hygro & LB1x62N HCL 2	1.57	71	0.1208
HSC-62N & LB1x62N HCL 1	1.70	60	0.0942
HSC-62N & LB1x62N HCL 2	2.35	53	0.0226

Figure AI-7: Mitomycin C dose response curves for cell lines from fusion 9. A. Responses of the control LCL, HSC-3TO (●), LB-1-hygro (▼) and the FA LCL from complementation group D, HSC-62N (▽); B. Responses of HSC-3TO (●), LB-1-hygro (▼), HSC-62N (▽), LB1x62N HCL 1 (□) and LB1x62N HCL 2 (■).


A
Relative Cell Number (%)
100
10
1
0
0
10
100
1000
Mitomycin C Dose (ng/mL)

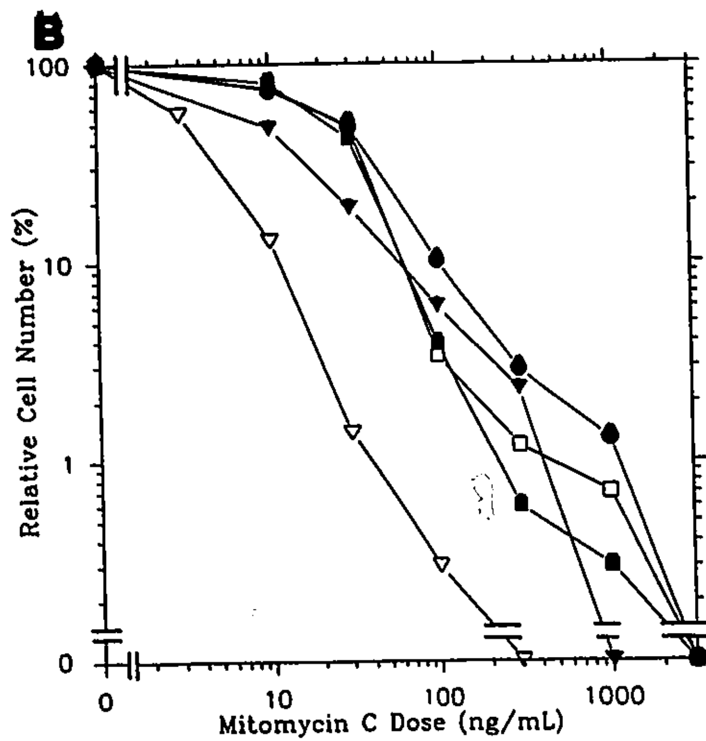

B
Relative Cell Number (%)
100
10
1
0
0
10
100
1000
Mitomycin C Dose (ng/mL)

Table AI-31: Results of Mitomycin C Dose Response Experiments - Fusion 9

MMC Dose ng/mL	Relative Cell Number (%) (sd)				
	HSC-3TO	HSC-62N	LB-1-hygro	LB1x62N HCL 1	LB1x62N HCL 2
0	100.0	100.0	100.0	100.0	100.0
3	- -	56.4 (15.1)	- -	- -	- -
10	74.2 (7.2)	12.9 (10.2)	47.8 (12.7)	76.2 (11.9)	80.0 (10.3)
30	48.5 (8.8)	1.4 (1.1)	18.8 (6.1)	49.5 (23.9)	42.5 (10.7)
100	10.2 (3.1)	0.3 (0.3)	6.0 (2.2)	3.4 (1.8)	3.9 (1.1)
300	2.9 (0.9)	0.0 (0.0)	2.3 (1.1)	1.2 (0.9)	0.6 (0.5)
1000	1.3 (0.7)	0.0 (0.0)	0.1 (0.2)	0.7 (0.7)	0.3 (0.4)
3000	0.0 (0.0)	- -	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

- = dose not used

Table AI-32: Statistical Analyses of Mean Mitomycin C D_{10} Values for HSC-3TO, LB-1-*hygro*, HSC-62N and LB-1x62N HCLs

(a) Analysis of Variance Table for D_{10}

Source	df	SS	MS	F	P
Cell Line	4	52451.00	13113.00	62.98	<0.0001
Experiment	2	4637.60	2318.80	11.14	0.0001
Replicate	3	480.48	160.16	0.77	0.5171
Error	46	9576.80	208.19		
Total	55	67146.00			
Grand Mean	1	191880.00			

(b) Tukey (WSD)* Pairwise Comparisons of Mean D_{10} (ng/mL) by Cell Line**

HSC-3TO	LB1x62N 1	LB1x62N 2	LB-1	HSC-62N
101.4	67.4	57.8	51.9	9.6

(Line under LB1x62N 1, LB1x62N 2 and LB-1)

* Wholly Significant Difference

** Groups which are not significantly different from each other are indicated by lines ($\alpha = 0.05$)

APPENDIX II:
Data from EMS Experiments

Figure AII-1: Relative cloning efficiency dose response curves for the control LCLs following treatment with EMS. DM (●), HSC-55 (○), GM1899A (▼), HSC-3TO (▽), JaKr (■) and PeKr (□).

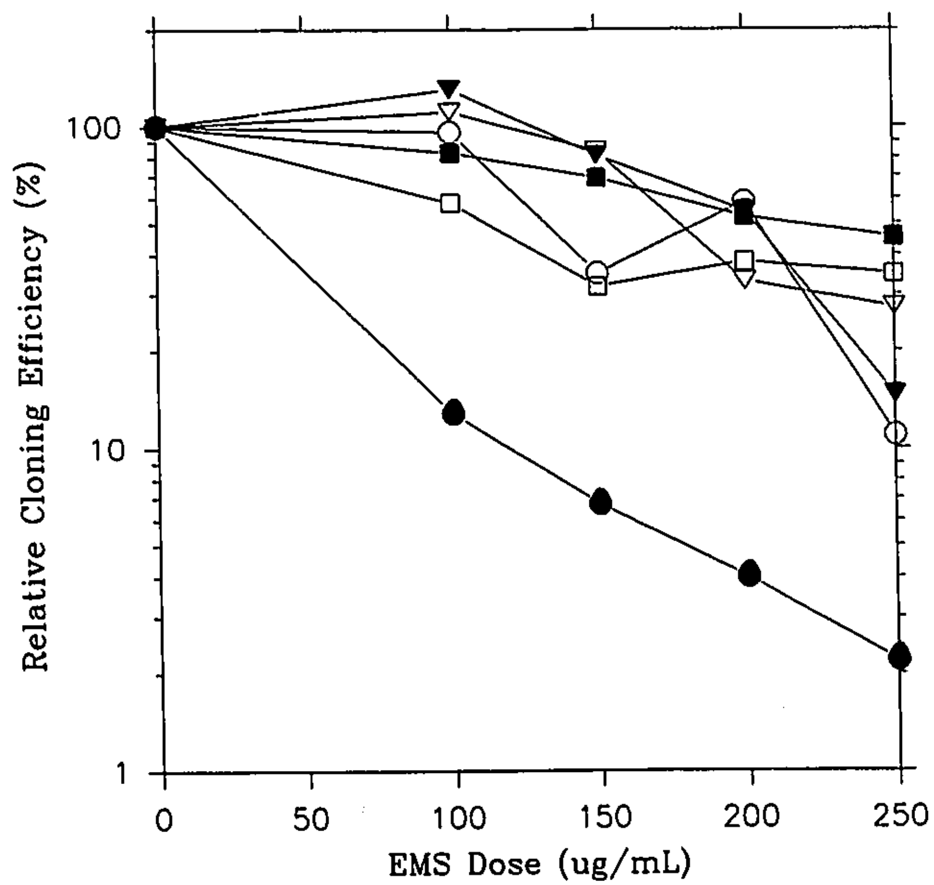
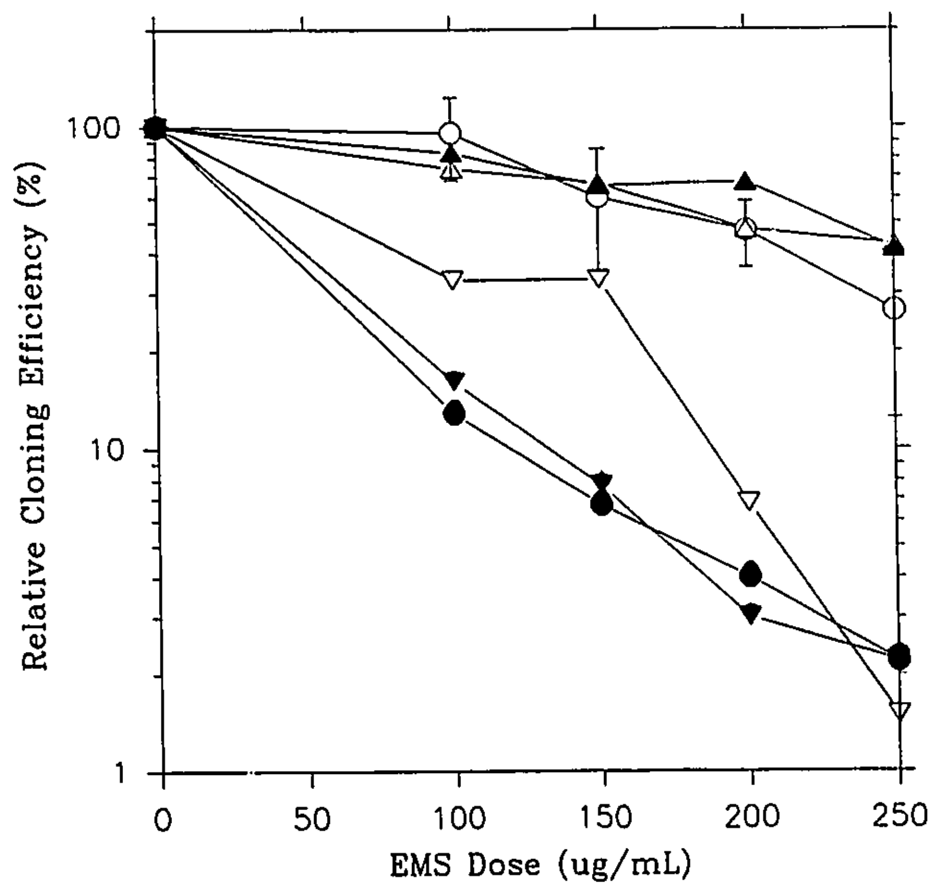

100
10
1
0
50
100
150
200
250
Relative Cloning Efficiency (%)
EMS Dose (ug/mL)

Figure AII-2: Relative cloning efficiency dose response curves following EMS treatment for DM (●), the mean of the other five control LCLs (O) (error bars represent one standard deviation), two RS- LCLs, TiBu (▲) and LoGi (△) and two RS+ LCLs, LB-1 (▽) and R20 (▼).



100
10
1
0
50
100
150
200
250
Relative Cloning Efficiency (%)
EMS Dose (ug/mL)

Figure AII-3: Relative cloning efficiency dose response curves following EMS treatment DM (●), the mean of the other five control LCLs (○) (error bars represent one standard deviation), two RS+ LCLs, LB-1 (▼) and R20 (▽) and two FA LCLs from complementation group A, HSC-72OT (▲) and HSC-99 (△).

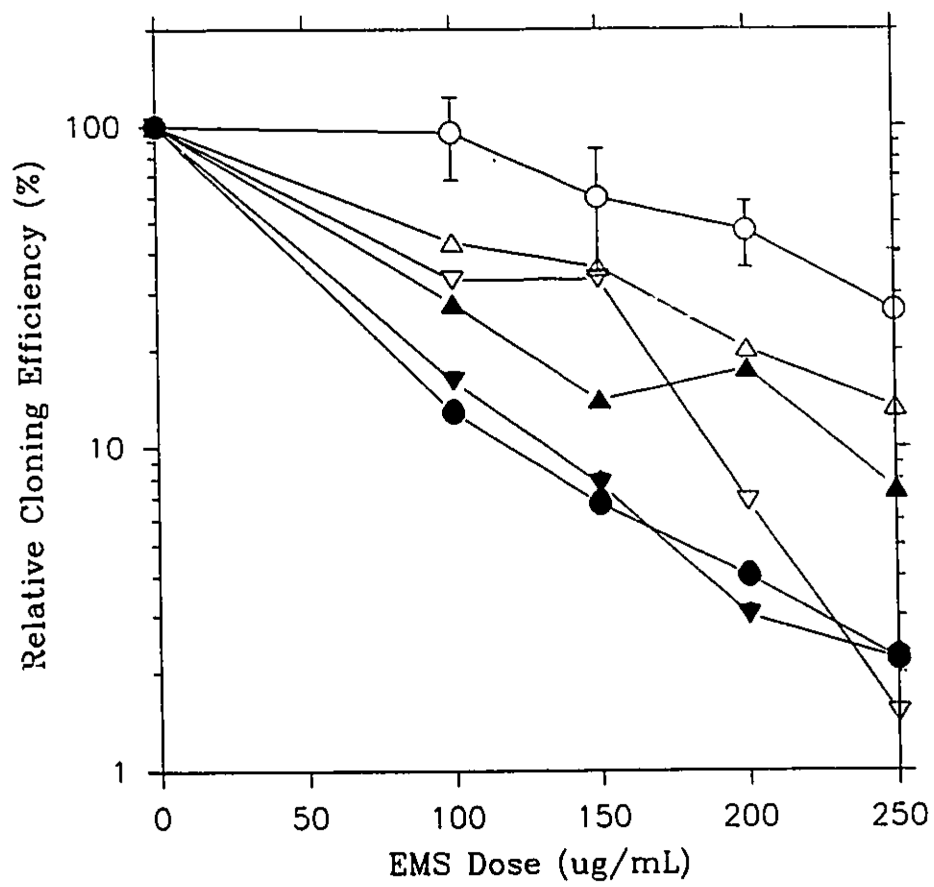
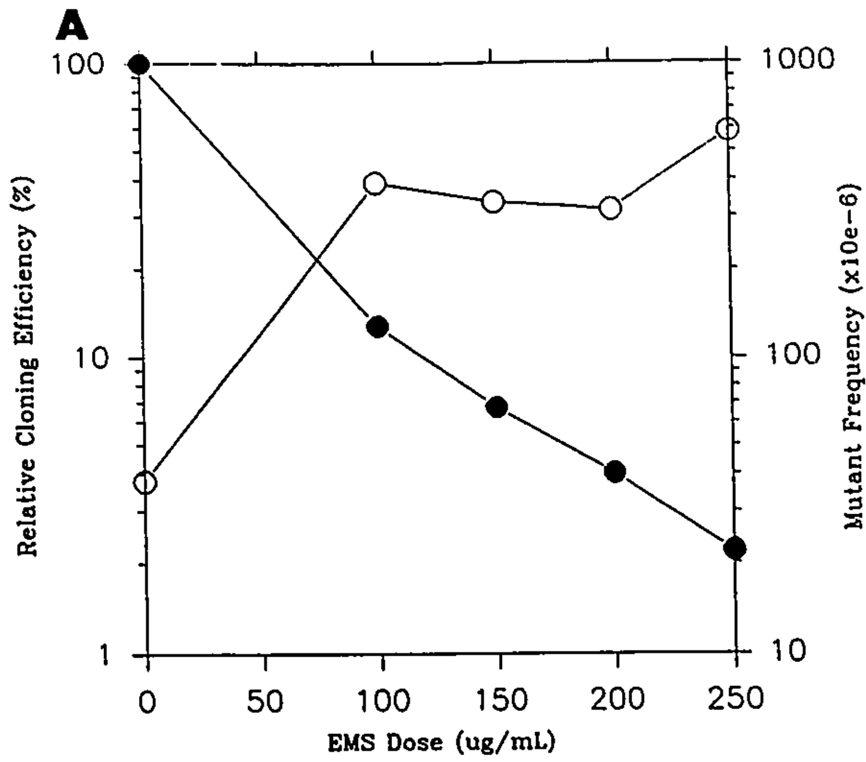

100
10
1
0
50
100
150
200
250
Relative Cloning Efficiency (%)
EMS Dose (ug/mL)

Figure AII-4: Cellular sensitivity and mutability of DM LCLs by EMS treatment in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (●) and the right vertical axis shows mutant frequency (O).


A
100
10
1
1000
100
10
0
50
100
150
200
250
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
EMS Dose (ug/mL)

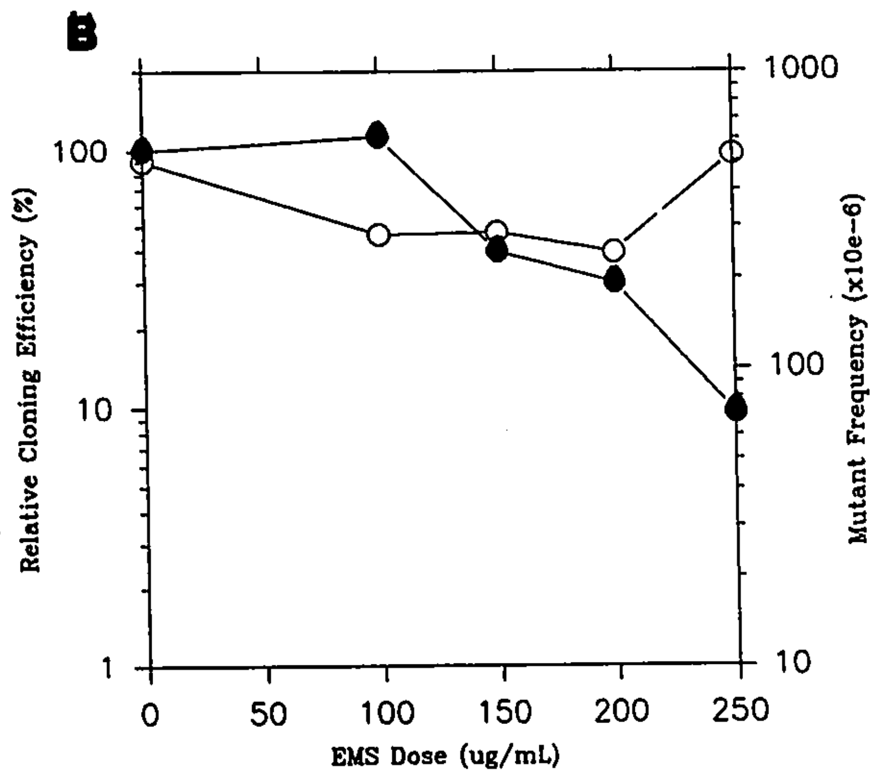

B
100
10
1
1000
100
10
0
50
100
150
200
250
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
EMS Dose (ug/mL)

Table AII-1: Summary of EMS Sensitivity and Mutability Data for DM in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-48b	0	0.0650	0.0140	0	0
	100	0.0040	0.0128	$1.2x10^{-6}$	$9.53x10^{-5}$
	150	0.0018	0.0086	$9.0x10^{-7}$	$1.10x10^{-4}$
	200	0.0017	0.0090	$1.4x10^{-6}$	$1.57x10^{-4}$
	250	0.0008	0.0065	$7.0x10^{-7}$	$1.16x10^{-4}$
D-48c	0	0.0294	0.0523	0	0
	100	0.0033	0.0255	$3.0x10^{-6}$	$1.18x10^{-4}$
	150	0.0017	0.0183	$2.9x10^{-6}$	$1.57x10^{-4}$
	200	0.0007	0.0258	$4.3x10^{-6}$	$1.65x10^{-4}$
	250	0.0008	0.0556	$4.2x10^{-6}$	$7.64x10^{-5}$
D-59	0	0.0358	0.0440	0	0
	100	0.0007	0.0374	$7.9x10^{-6}$	$2.10x10^{-4}$
	150	0.0002	0.0369	$3.1x10^{-6}$	$8.36x10^{-4}$
	200	0.0000	0.0453	$3.7x10^{-5}$	$8.13x10^{-4}$
	250	0.0000	0.0286	$2.9x10^{-6}$	$1.02x10^{-4}$
D-62	0	0.0408	0.0117	0	0
	100	0.0002	0.0040	$3.8x10^{-6}$	$9.55x10^{-4}$
	150	0.0003	0.0044	$4.7x10^{-6}$	$1.10x10^{-3}$
	200	0.0000	0.0088	$2.1x10^{-6}$	$2.39x10^{-4}$
	250	0.0000	0.0069	$6.5x10^{-6}$	$9.51x10^{-4}$
D-69b	0	0.0129	0.0044	0	0
	100	0.0000	0.0049	$3.6x10^{-6}$	$7.29x10^{-4}$
	150	0.0000	0.0063	$3.5x10^{-6}$	$5.57x10^{-4}$
	200	0.0000	0.0171	$1.3x10^{-6}$	$7.47x10^{-5}$
	250	0.0000	0.0147	$3.7x10^{-5}$	$2.51x10^{-3}$
D-76	0	0.0258	0.0307	$4.9x10^{-6}$	$1.59x10^{-6}$
	100	0.0010	0.0149	$4.4x10^{-6}$	$2.98x10^{-4}$
	150	0.0002	0.0298	$5.3x10^{-6}$	$1.78x10^{-4}$

	150	0.0003	0.0044	4.7×10^{-6}	1.10×10^{-3}
	200	0.0000	0.0088	2.1×10^{-6}	2.39×10^{-4}
	250	0.0000	0.0069	6.5×10^{-6}	9.51×10^{-4}
D-69b	0	0.0129	0.0044	0	0
	100	0.0000	0.0049	3.6×10^{-6}	7.29×10^{-4}
	150	0.0000	0.0063	3.5×10^{-6}	5.57×10^{-4}
	200	0.0000	0.0171	1.3×10^{-6}	7.47×10^{-5}
	250	0.0000	0.0147	3.7×10^{-5}	2.51×10^{-3}
D-76	0	0.0258	0.0307	4.9×10^{-6}	1.59×10^{-6}
	100	0.0010	0.0149	4.4×10^{-6}	2.98×10^{-4}
	150	0.0002	0.0298	5.3×10^{-6}	1.78×10^{-4}
	200	0.0000	0.0217	6.0×10^{-6}	2.78×10^{-4}
	250	0.0000	0.0298	8.6×10^{-6}	2.88×10^{-4}
D-80b	0	0.0053	0.0280	3.3×10^{-8}	1.17×10^{-6}
	100	0.0000	0.0223	1.3×10^{-5}	5.77×10^{-4}
	150	0.0000	0.0144	5.1×10^{-6}	3.56×10^{-4}
	200	0.0000	0.0129	7.7×10^{-6}	5.98×10^{-4}
	250	0.0000	0.0190	9.1×10^{-6}	4.81×10^{-4}
D-133	0	0.0278	0.0255	4.7×10^{-6}	1.83×10^{-4}
	100	0.0315	0.0127	1.8×10^{-6}	1.43×10^{-4}
	150	0.0110	0.0106	1.4×10^{-6}	1.34×10^{-4}
	200	0.0083	0.0130	2.5×10^{-6}	1.95×10^{-4}
	250	0.0027	0.0196	2.5×10^{-6}	1.30×10^{-4}

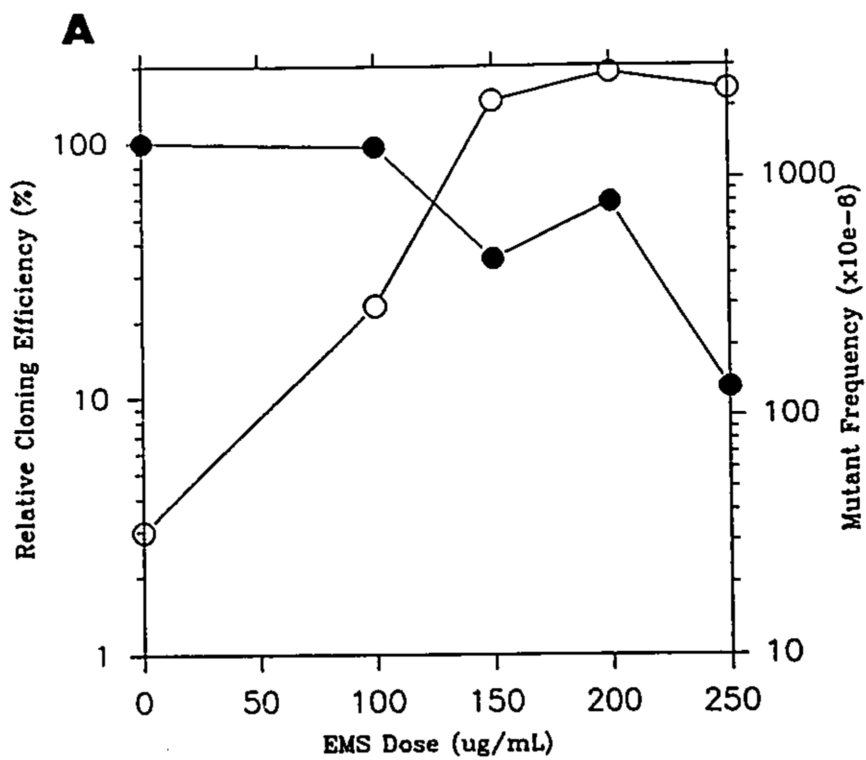
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-2: Summary of EMS Sensitivity and Mutability Data for DM in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-133 (TK6)	0	0.0150	0.0103	$5.2x10^{-6}$	$5.02x10^{-4}$
	100	0.0118	0.0099	$2.8x10^{-6}$	$2.81x10^{-4}$
	150	0.0065	0.0044	$1.3x10^{-6}$	$2.85x10^{-4}$
	200	0.0040	0.0080	$1.9x10^{-6}$	$2.43x10^{-4}$
	250	0.0020	0.0055	$2.9x10^{-6}$	$5.31x10^{-4}$

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-5: Cellular sensitivity and mutability of HSC-55 LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (●) and the right vertical axis shows mutant frequency (O).


A
Relative Cloning Efficiency (%)
100
10
1
Mutant Frequency (x10e-6)
1000
100
10
0
50
100
150
200
250
EMS Dose (ug/mL)

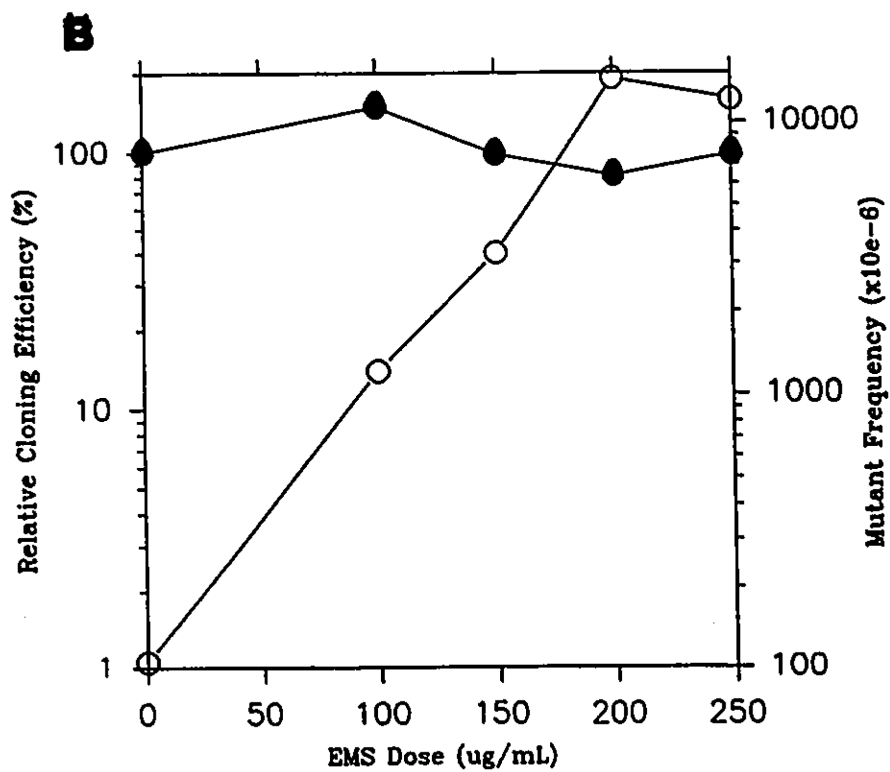

B
Relative Cloning Efficiency (%)
100
10
1
Mutant Frequency (x10e-6)
10000
1000
100
0
50
100
150
200
250
EMS Dose (ug/mL)

Table AII-3: Summary of EMS Sensitivity and Mutability Data for HSC-55 in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-115	0	0.00287	0.00134	4.3×10^{-8}	3.24×10^{-5}
	100	0.00275	0.00271	7.9×10^{-7}	2.93×10^{-4}
	150	0.00099	0.00082	1.8×10^{-6}	2.13×10^{-3}
	200	0.00167	0.00132	3.7×10^{-6}	2.81×10^{-3}
	250	0.00033	0.00133	3.1×10^{-6}	2.37×10^{-3}

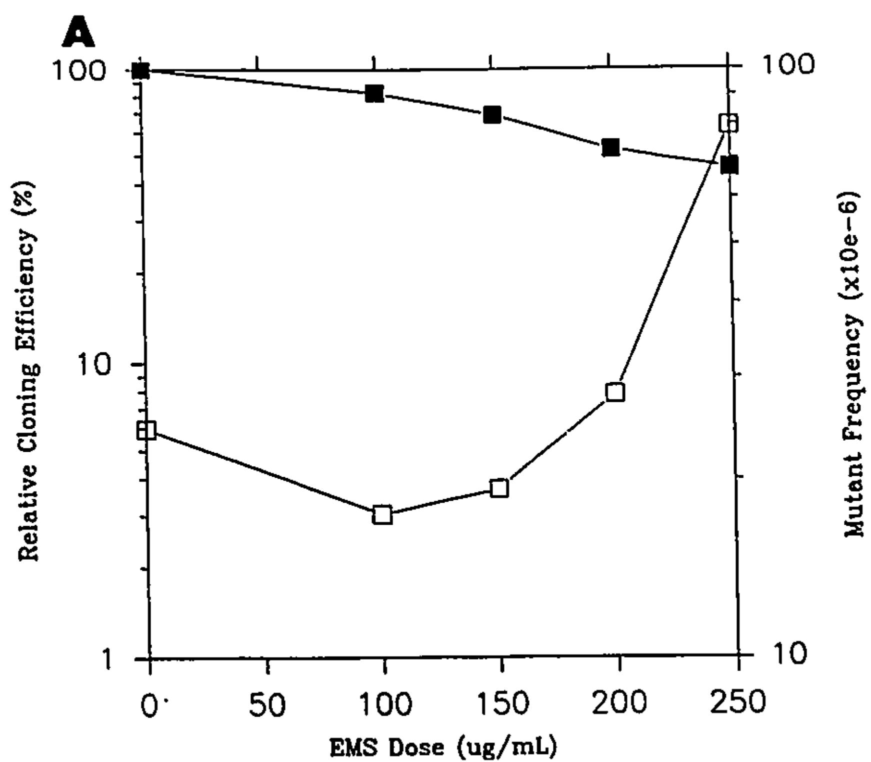
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-4: Summary of EMS Sensitivity and Mutability Data for HSC-55 in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-115 (TK6)	0	0.00101	0.000496	5.2×10^{-8}	1.05×10^{-4}
	100	0.00150	0.000656	7.9×10^{-5}	1.21×10^{-3}
	150	0.00099	0.000665	2.2×10^{-6}	3.27×10^{-3}
	200	0.00082	0.000164	2.4×10^{-6}	1.44×10^{-2}
	250	0.00099	0.000327	3.9×10^{-6}	1.21×10^{-2}

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-6: Cellular sensitivity and mutability of JaKr LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (■) and the right vertical axis shows mutant frequency (□).


A
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
100
10
0
50
100
150
200
250
EMS Dose (ug/mL)

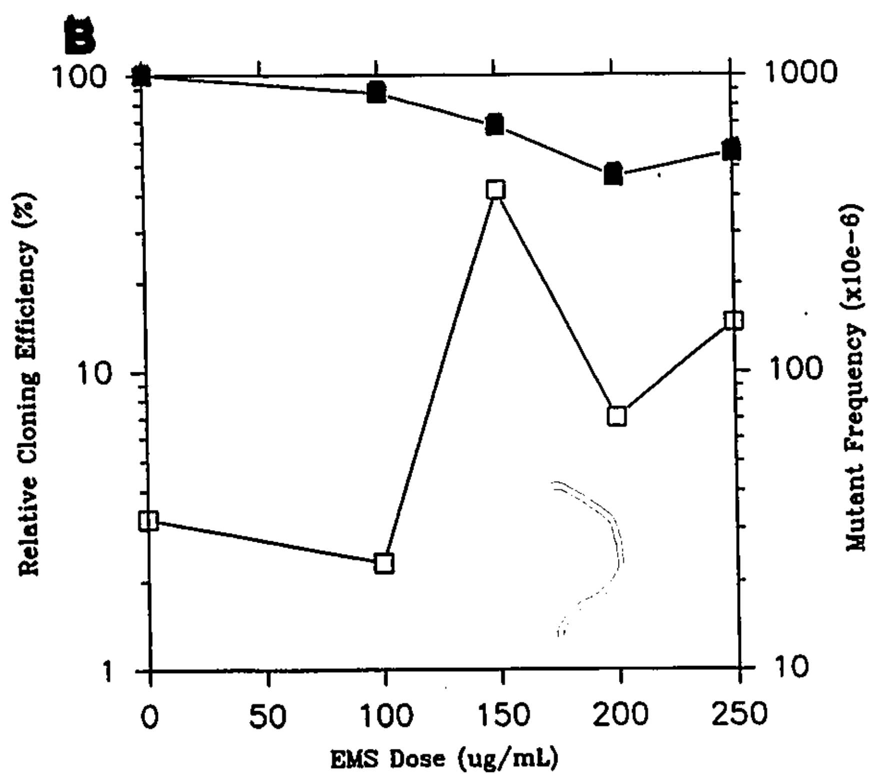

B
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
1000
100
10
0
50
100
150
200
250
EMS Dose (ug/mL)

Table AII-5: Summary of EMS Sensitivity and Mutability Data for JaKr in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-80b	0	0.0570	0.1450	1.9×10^{-7}	1.26×10^{-6}
	100	0.0540	0.0740	2.0×10^{-6}	2.67×10^{-5}
	150	0.0390	0.0692	1.2×10^{-6}	1.73×10^{-5}
	200	0.0315	0.0677	2.4×10^{-6}	3.64×10^{-5}
	250	0.2660	0.0993	3.8×10^{-6}	3.83×10^{-5}
D-85	0	0.0750	0.1100	6.0×10^{-7}	5.62×10^{-6}
	100	0.0760	0.0866	5.5×10^{-7}	6.31×10^{-6}
	150	0.0460	0.0980	1.1×10^{-6}	1.13×10^{-5}
	200	0.0340	0.0996	1.3×10^{-6}	1.26×10^{-5}
	250	0.0220	0.0759	3.8×10^{-6}	5.03×10^{-5}
D-105b	0	0.1020	0.0243	1.6×10^{-6}	6.62×10^{-5}
	100	0.0054	0.0224	4.3×10^{-7}	1.91×10^{-5}
	150	0.0079	0.0193	5.6×10^{-7}	2.90×10^{-5}
	200	0.0058	0.0342	1.2×10^{-6}	3.50×10^{-5}
	250	0.0062	0.0216	3.2×10^{-6}	1.50×10^{-4}

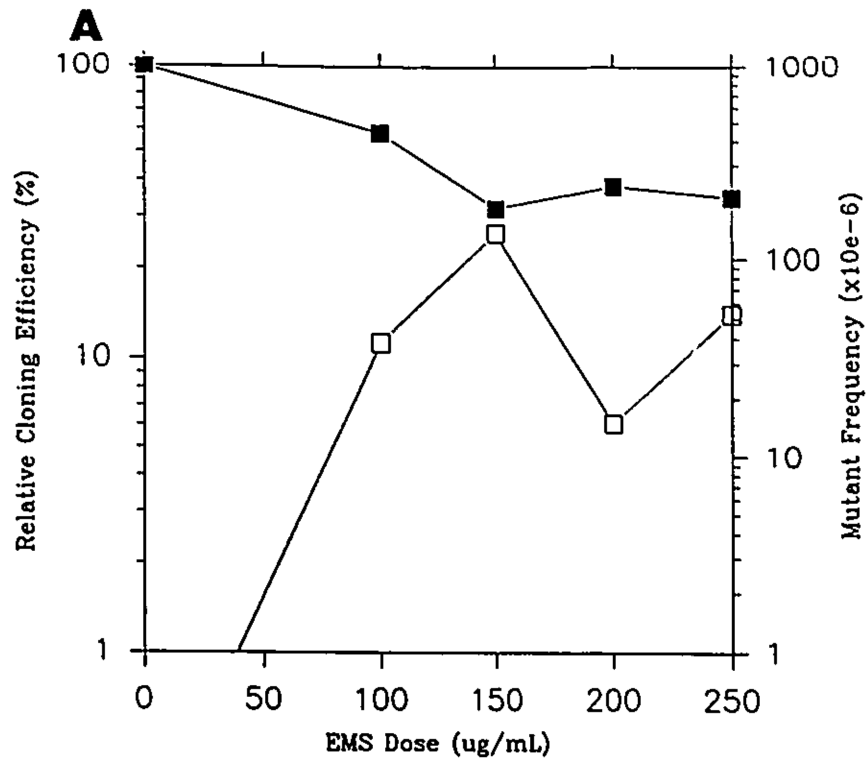
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-6: Summary of EMS Sensitivity and Mutability Data for JaKr in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-105 (TK6)	0	0.0026	0.0040	0	0
	100	0.0020	0.0033	$3.1x10^{-8}$	$9.20x10^{-6}$
	150	0.0081	0.0074	$5.7x10^{-6}$	$7.75x10^{-4}$
	200	0.0033	0.0033	$2.0x10^{-7}$	$8.35x10^{-5}$
	250	0.0040	0.0040	$1.0x10^{-7}$	$3.94x10^{-5}$
D-105b (TK6)	0	0.0051	0.0280	$1.8x10^{-6}$	$6.43x10^{-5}$
	100	0.0054	0.0134	$4.9x10^{-7}$	$3.69x10^{-5}$
	150	0.0047	0.0099	$5.6x10^{-7}$	$5.68x10^{-5}$
	200	0.0030	0.0239	$1.3x10^{-6}$	$5.60x10^{-5}$
	250	0.0040	0.0114	$2.9x10^{-6}$	$2.54x10^{-4}$

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-7: Cellular sensitivity and mutability of PeKr LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (■) and the right vertical axis shows mutant frequency (□).


A
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
1000
100
10
1
0
50
100
150
200
250
EMS Dose (ug/mL)

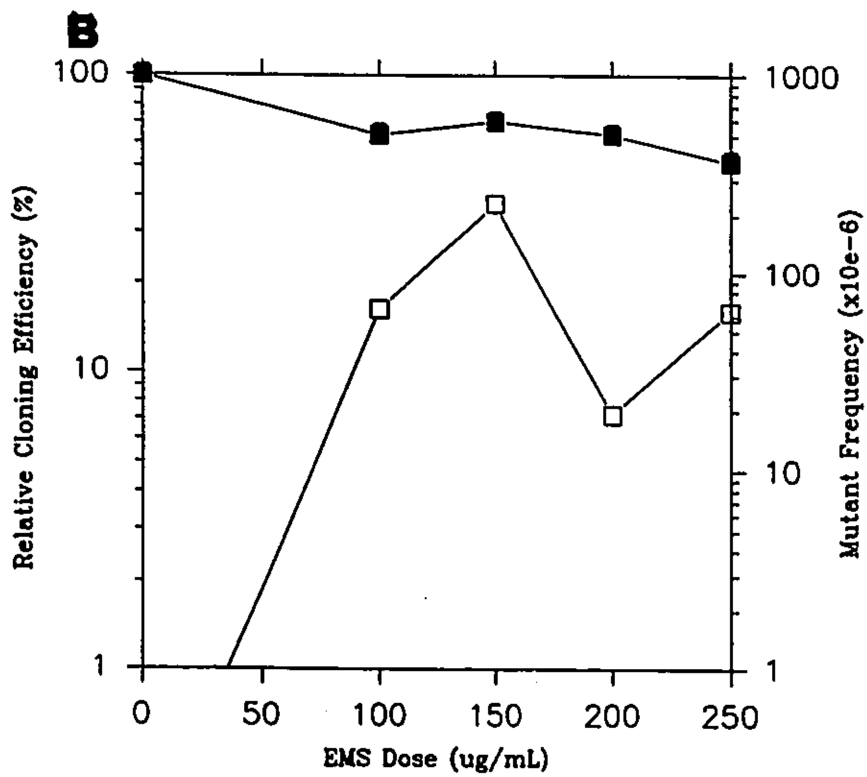

B
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
1000
100
10
1
0
50
100
150
200
250
EMS Dose (ug/mL)

Table AII-7: Summary of EMS Sensitivity and Mutability Data for PeKr in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-105b	0	0.0106	0.0047	0	0
	100	0.0062	0.0084	3.1×10^{-7}	3.76×10^{-5}
	150	0.0033	0.0040	5.4×10^{-7}	1.34×10^{-4}
	200	0.0040	0.0062	9.2×10^{-8}	1.49×10^{-5}
	250	0.0037	0.0076	4.0×10^{-7}	5.28×10^{-5}

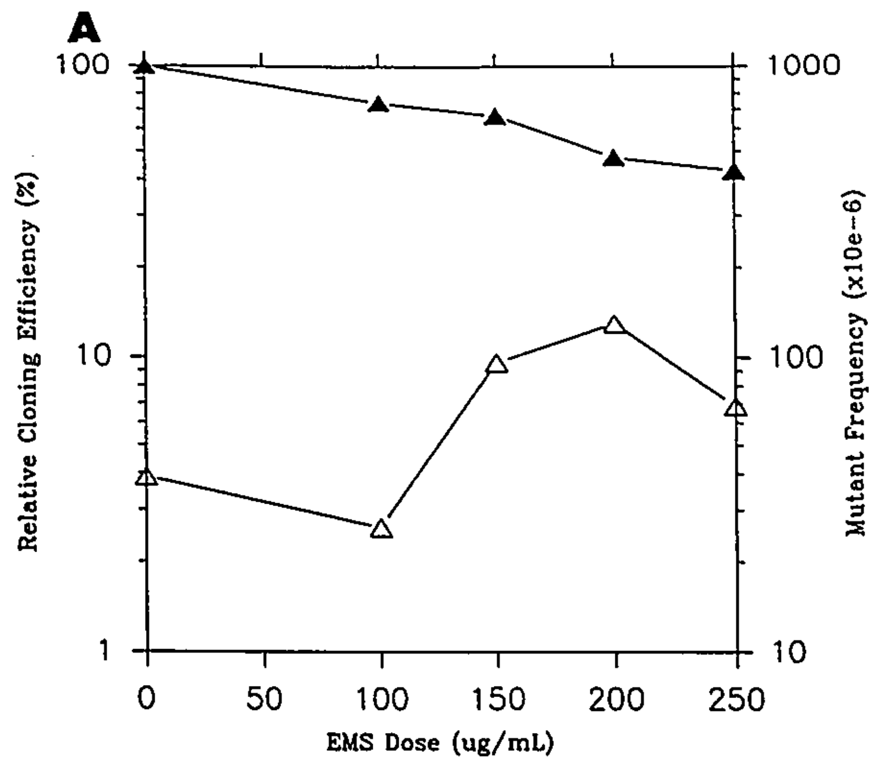
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-8: Summary of EMS Sensitivity and Mutability Data for PeKr in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-105 (TK6)	0	0.0145	0.0074	0	0
	100	0.0081	0.0053	$5.2x10^{-8}$	$9.80x10^{-6}$
	150	0.0102	0.0074	$5.0x10^{-7}$	$6.80x10^{-5}$
	200	0.0123	0.0074	$7.8x10^{-8}$	$1.06x10^{-5}$
	250	0.0088	0.0040	$1.0x10^{-7}$	$4.23x10^{-5}$
D-105b (TK6)	0	0.0023	0.0023	0	0
	100	0.0017	0.0030	$2.0x10^{-7}$	$6.53x10^{-5}$
	150	no data	0.0017	$3.8x10^{-7}$	$2.30x10^{-4}$
	200	0.0010	0.0054	$1.0x10^{-7}$	$1.92x10^{-5}$
	250	0.0010	0.0017	$1.0x10^{-7}$	$6.33x10^{-5}$

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-8: Cellular sensitivity and mutability of LoGi LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (▲) and the right vertical axis shows mutant frequency (△).


A
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
EMS Dose (ug/mL)
100
10
1
1000
100
10
0
50
100
150
200
250

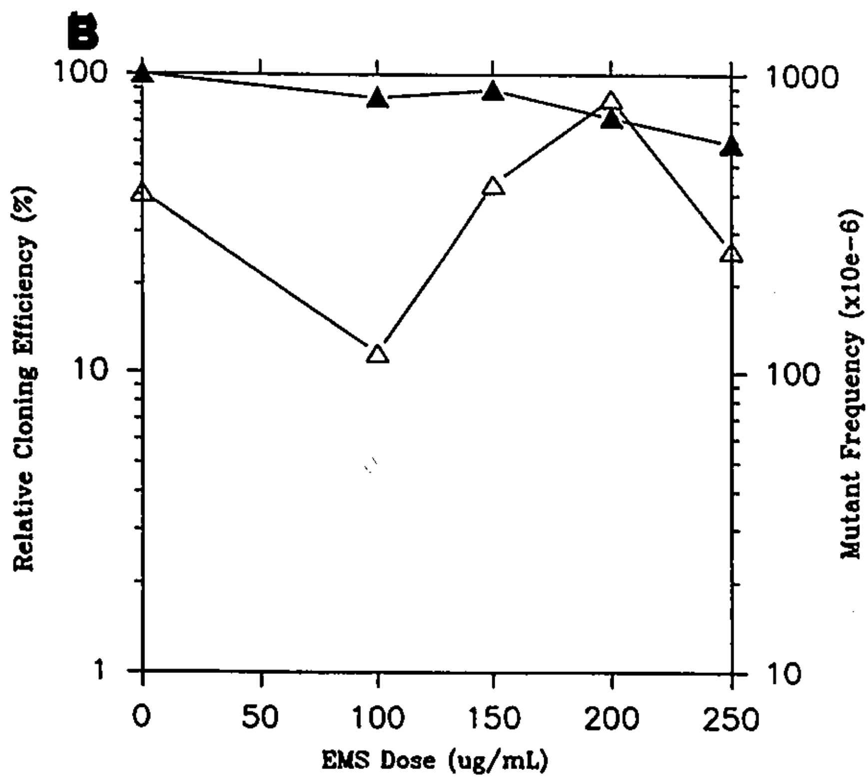

B
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
EMS Dose (ug/mL)
100
10
1
1000
100
10
0
50
100
150
200
250

Table AII-9: Summary of EMS Sensitivity and Mutability Data for LoGi in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-76	0	0.0177	0.0280	$1.8x10^{-7}$	$6.45x10^{-6}$
	100	0.0124	0.0262	$7.2x10^{-7}$	$2.74x10^{-5}$
	150	0.0134	0.0195	$9.4x10^{-7}$	$4.82x10^{-5}$
	200	0.0115	0.0210	$6.8x10^{-7}$	$3.24x10^{-5}$
	250	0.0094	0.0192	$1.9x10^{-6}$	$1.01x10^{-4}$
D-115	0	0.0228	0.0111	$7.9x10^{-7}$	$7.12x10^{-5}$
	100	0.0177	0.0191	$4.7x10^{-7}$	$2.48x10^{-5}$
	150	0.0130	0.0074	$1.2x10^{-6}$	$1.59x10^{-4}$
	200	0.0068	0.0058	$1.3x10^{-6}$	$2.26x10^{-4}$
	250	0.0029	0.0131	$4.7x10^{-7}$	$3.61x10^{-5}$

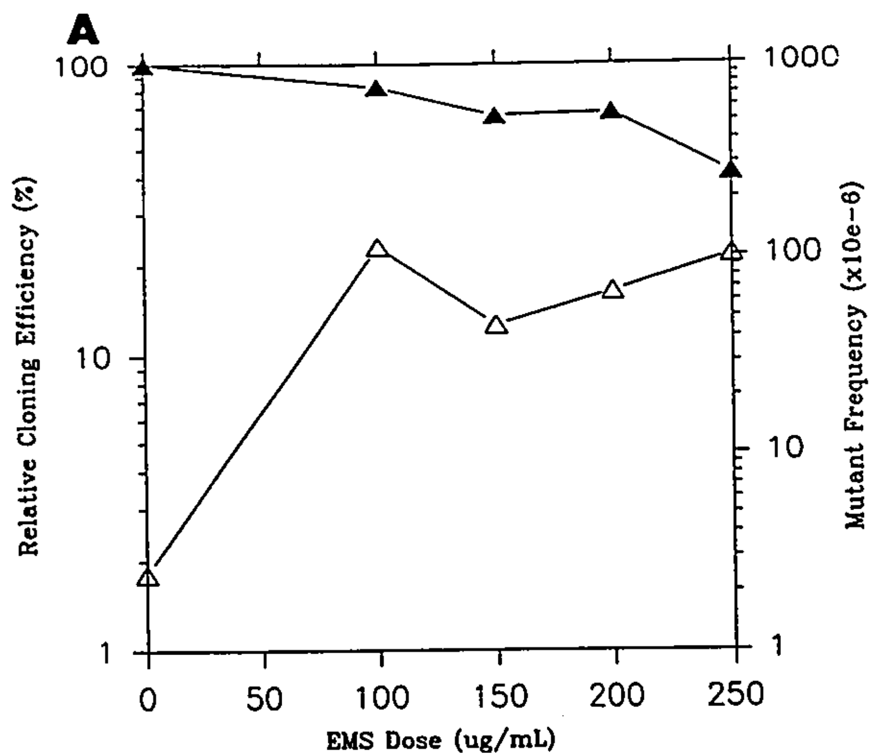
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-10: Summary of EMS Sensitivity and Mutability Data for LoGi in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-115 (TK6)	0	0.0134	0.0018	$7.4x10^{-7}$	$4.05x10^{-4}$
	100	0.0112	0.0050	$5.8x10^{-7}$	$1.16x10^{-4}$
	150	0.0119	0.0044	$1.9x10^{-6}$	$4.33x10^{-4}$
	200	0.0096	0.0008	$6.9x10^{-7}$	$8.34x10^{-4}$
	250	0.0080	0.0015	$3.9x10^{-7}$	$2.61x10^{-4}$

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-9: Cellular sensitivity and mutability of TiBu LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (▲) and the right vertical axis shows mutant frequency (△).


A
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
1000
100
10
1
0
50
100
150
200
250
EMS Dose (ug/mL)

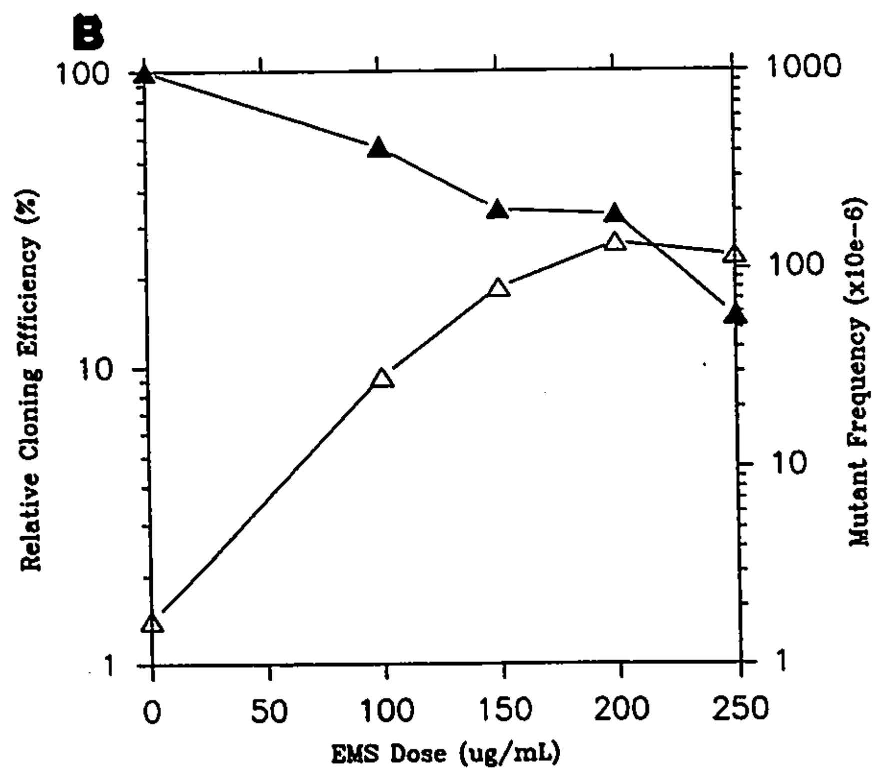

B
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
1000
100
10
1
0
50
100
150
200
250
EMS Dose (ug/mL)

Table AII-11: Summary of EMS Sensitivity and Mutability Data for TiBu in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-69b	0	0.0088	0.0119	$2.9x10^{-8}$	$2.43x10^{-6}$
	100	0.0057	0.0088	$9.8x10^{-7}$	$1.11x10^{-4}$
	150	0.0044	0.0088	$3.9x10^{-7}$	$4.49x10^{-5}$
	200	0.0044	0.0177	$1.1x10^{-6}$	$6.63x10^{-5}$
	250	0.0025	0.0105	$1.1x10^{-6}$	$1.02x10^{-4}$

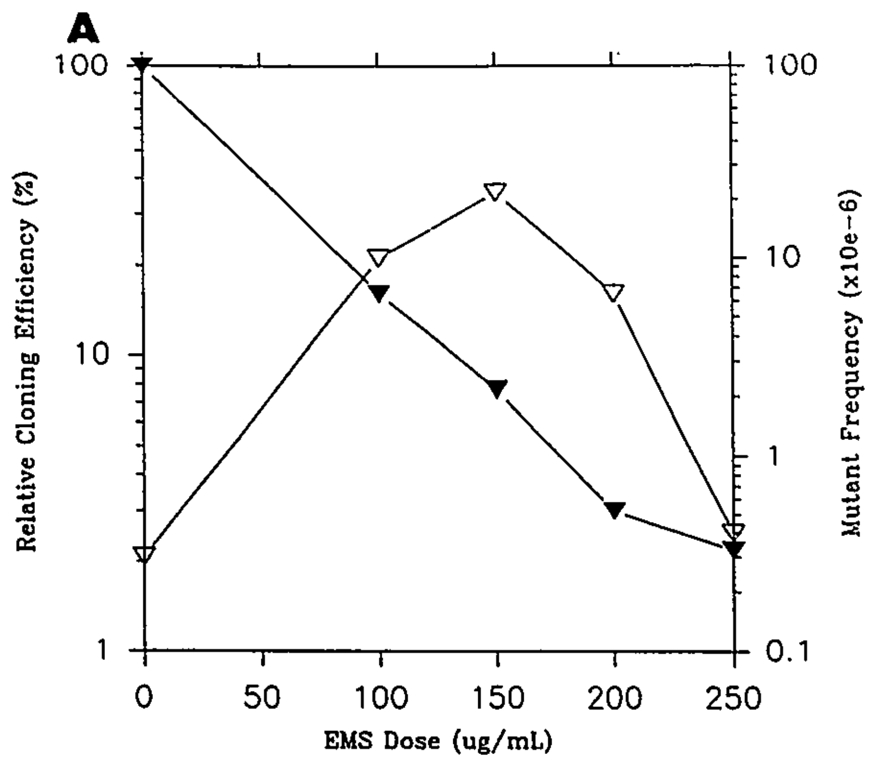
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-12: Summary of EMS Sensitivity and Mutability Data for TiBu in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-97 (TK6)	0	0.0320	0.0433	$7.2x10^{-8}$	$1.67x10^{-6}$
	100	0.0180	0.0371	$1.0x10^{-6}$	$2.87x10^{-5}$
	150	0.0110	0.0481	$3.8x10^{-6}$	$7.96x10^{-5}$
	200	0.0106	0.0239	$3.2x10^{-6}$	$1.37x10^{-4}$
	250	0.0047	0.0495	$5.7x10^{-6}$	$1.16x10^{-4}$

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-10: Cellular sensitivity and mutability of LB-1 LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (▼) and the right vertical axis shows mutant frequency (▽). Note that the scale for mutant frequency in (A) and (B) is an order of magnitude lower than in graphs for control, RS- and FA LCLs.


A
100
10
1
100
10
1
0.1
0
50
100
150
200
250
EMS Dose (ug/mL)
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)

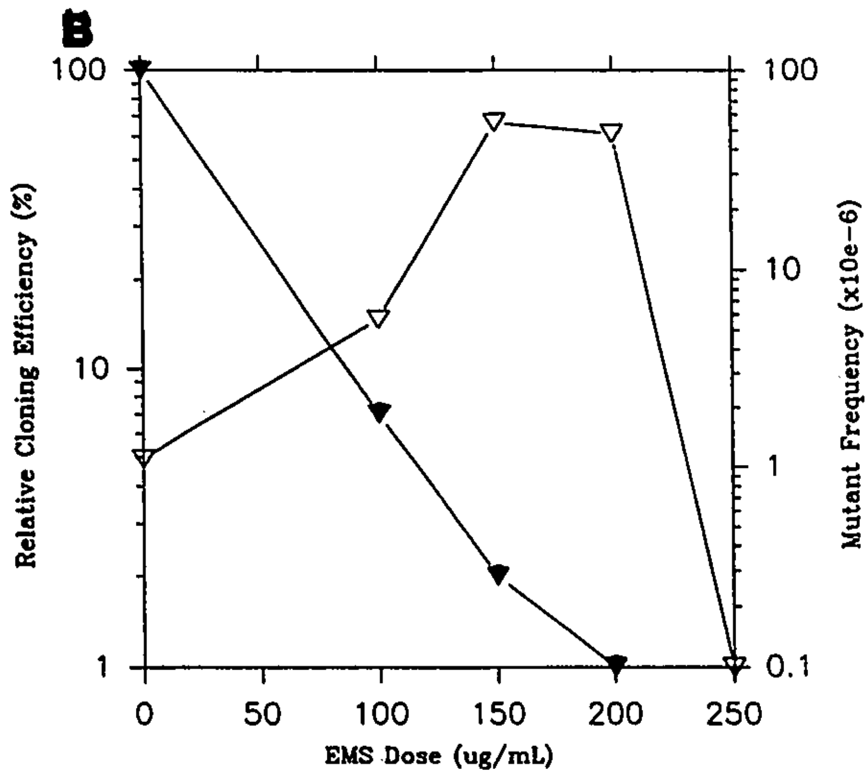

B
100
10
1
100
10
1
0.1
0
50
100
150
200
250
EMS Dose (ug/mL)
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)

Table AII-13: Summary of EMS Sensitivity and Mutability Data for LB-1 in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-48a	0	0.0086	0.0072	0	0
	100	0.0003	0.0112	0	0
	150	0.0003	0.0096	0	0
	200	0.0000	0.0122	0	0
	250	0.0000	0.0052	0	0
D-48b	0	0.0147	0.0116	0	0
	100	0.0035	0.0054	$2.0x10^{-7}$	$4.12x10^{-5}$
	150	0.0027	0.0158	$3.7x10^{-8}$	$2.36x10^{-4}$
	200	0.0016	0.0105	0	0
	250	0.0016	0.0098	0	0
D-62	0	0.0210	0.0134	0	0
	100	0.0033	0.0088	0	0
	150	0.0010	0.0049	0	0
	200	0.0005	0.0075	0	0
	250	0.0000	0.0020	0	0
D-85	0	0.0018	0.0007	0	0
	100	0.0003	0.0017	0	0
	150	0.0000	0.0013	0	0
	200	0.0000	no data	no data	no data
	250	0.0000	no data	no data	no data
D-134	0	0.0113	0.0214	$2.9x10^{-8}$	$1.36x10^{-6}$
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
	150	0.0010	0.0049	0	0
	200	0.0005	0.0075	0	0
	250	0.0000	0.0020	0	0
D-85	0	0.0018	0.0007	0	0
	100	0.0003	0.0017	0	0
	150	0.0000	0.0013	0	0
	200	0.0000	no data	no data	no data
	250	0.0000	no data	no data	no data
D-134	0	0.0113	0.0214	$2.9x10^{-8}$	$1.36x10^{-6}$
	100	0.0016	0.0250	$2.0x10^{-7}$	$7.88x10^{-6}$
	150	0.0014	0.0104	$1.1x10^{-6}$	$1.03x10^{-4}$
	200	0.0002	0.0189	$4.9x10^{-7}$	$2.60x10^{-5}$
	250	0.0000	0.0194	$3.3x10^{-8}$	$1.69x10^{-6}$

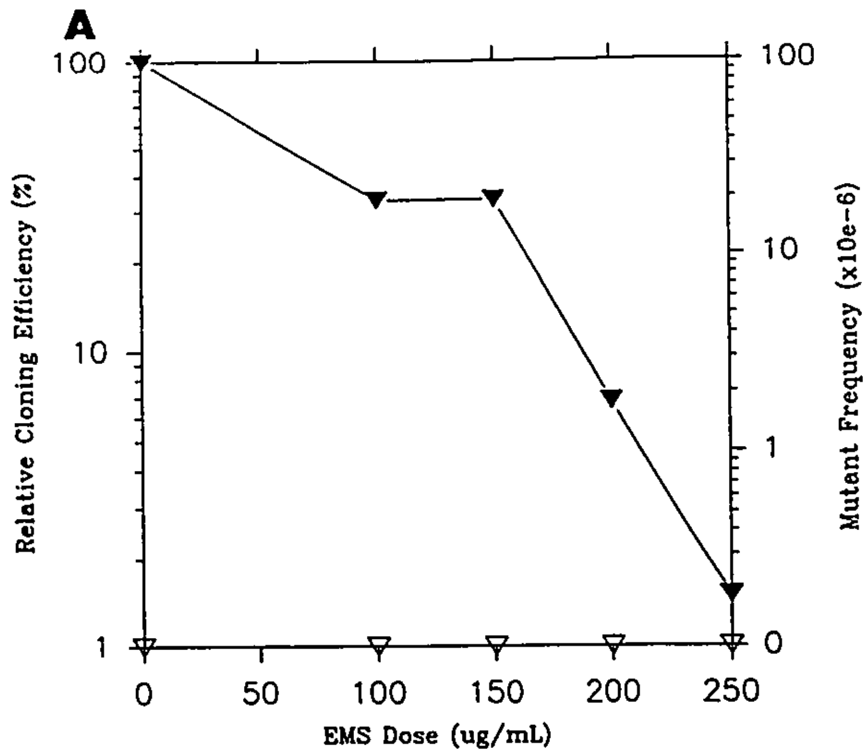
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-14: Summary of EMS Sensitivity and Mutability Data for LB-1 in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-100 (TK6)	0	0.0023	0.0038	0	0
	100	0.0000	0.0026	0	0
	150	0.0000	0.0021	$2.0x10^{-8}$	$9.30x10^{-6}$
	200	0.0000	0.0023	$3.6x10^{-8}$	$1.59x10^{-5}$
	250	0.0000	0.0713	0	0
D-134 (TK6)	0	0.0083	0.0149	$3.3x10^{-8}$	$2.20x10^{-6}$
	100	0.0058	0.0145	$1.6x10^{-7}$	$1.13x10^{-5}$
	150	0.0002	0.0076	$7.6x10^{-7}$	$1.00x10^{-4}$
	200	0.0001	0.0113	$9.1x10^{-7}$	$8.05x10^{-5}$
	250	0.0000	0.0076	0	0

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-11: Cellular sensitivity and mutability of R20 LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (▼) and the right vertical axis shows mutant frequency (▽). Note that the mutant frequency in the absence of a feeder layer is zero at all doses.


A
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
100
10
1
0
0
50
100
150
200
250
EMS Dose (ug/mL)

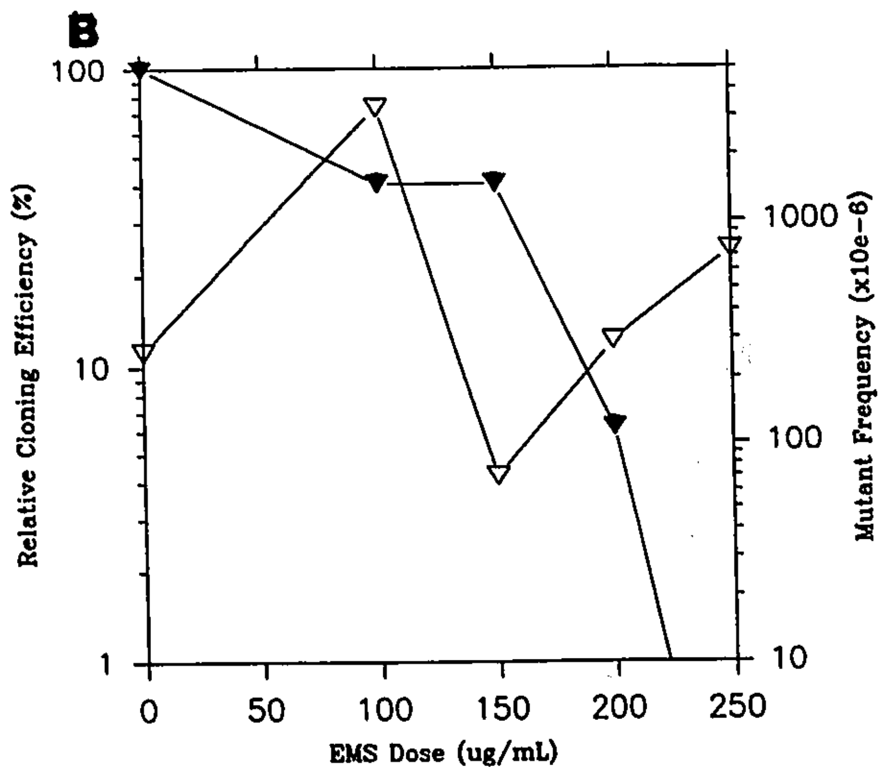

B
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
100
10
1
1000
100
10
0
50
100
150
200
250
EMS Dose (ug/mL)

Table AII-15: Summary of EMS Sensitivity and Mutability Data for R20 in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.*
D-48a	0	0.0083	0.0018	0	$<1.18\times10^{-5}$
	100	0.0023	0.0003	0	$<3.83\times10^{-4}$
	150	0.0020	0.0003	0	$<7.72\times10^{-5}$
	200	0.0005	0.0010	0	$<1.45\times10^{-4}$
	250	0.0003	0.0030	0	$<6.90\times10^{-6}$
D-48c	0	0.0115	0.0102	0	$<2.04\times10^{-5}$
	100	0.0038	0.0098	0	$<1.53\times10^{-4}$
	150	0.0038	0.0113	0	$<1.53\times10^{-4}$
	200	0.0008	0.0085	0	$<3.68\times10^{-5}$
	250	0.0002	0.0091	0	$<1.26\times10^{-5}$
D-59	0	0.0044	0.0012	0	$<3.20\times10^{-6}$
	100	0.0017	0.0025	0	$<1.40\times10^{-6}$
	150	0.0018	0.0055	0	$<6.00\times10^{-7}$
	200	0.0003	0.0008	0	$<4.50\times10^{-6}$
	250	0.0000	0.0031	0	$<1.20\times10^{-6}$

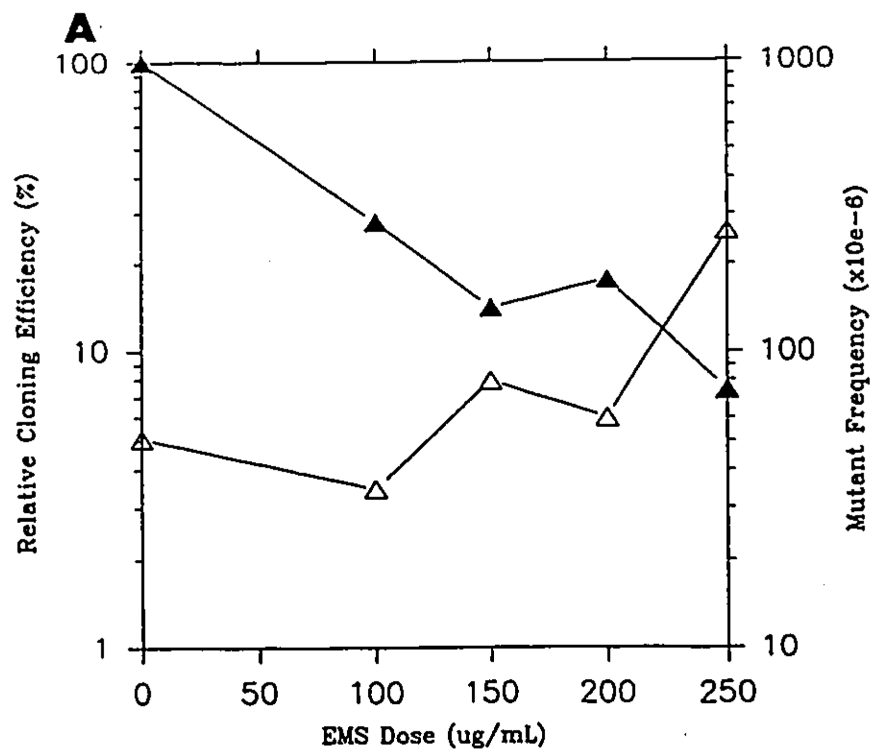
Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f.* = minimum mutant frequency based on <1 mutant isolated

Table AII-16: Summary of EMS Sensitivity and Mutability Data for R20 in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-91 (TK6)	0	0.0016	0.0052	0	0
	100	0.0005	0.0028	$3.0x10^{-7}$	$1.27x10^{-4}$
	150	0.0005	0.0022	$1.0x10^{-7}$	$6.98x10^{-5}$
	200	0.0000	0.0010	$5.0x10^{-7}$	$4.97x10^{-4}$
	250	0.0000	0.0011	0	0
D-97 (TK6)	0	0.0007	0.0023	$1.1x10^{-6}$	$5.22x10^{-4}$
	100	0.0003	0.0021	$1.4x10^{-5}$	$6.48x10^{-3}$
	150	0.0003	no data	no data	no data
	200	0.0001	0.0015	$1.2x10^{-6}$	$8.65x10^{-4}$
	250	0.0000	0.0014	$2.0x10^{-6}$	$1.48x10^{-3}$

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Figure AII-12: Cellular sensitivity and mutability of HSC-99 LCLs by EMS in the absence (A) and the presence (B) of an irradiated TK6 feeder layer. The left vertical axis shows relative cloning efficiency (▲) and the right vertical axis shows mutant frequency (Δ).


A
100
10
1
1000
100
10
0
50
100
150
200
250
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
EMS Dose (ug/mL)

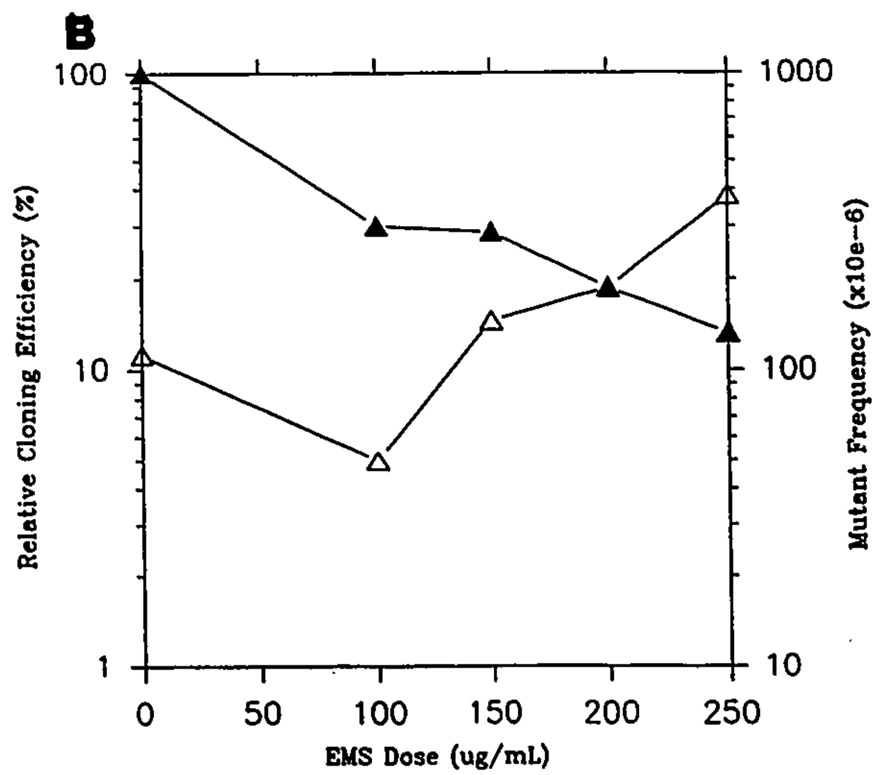

B
100
10
1
1000
100
10
0
50
100
150
200
250
Relative Cloning Efficiency (%)
Mutant Frequency (x10e-6)
EMS Dose (ug/mL)

Table AII-17: Summary of EMS Sensitivity and Mutability Data for HSC-99 in the Absence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-133	0	0.0304	0.0203	1.0×10^{-6}	5.07×10^{-5}
	100	0.0138	0.0209	7.2×10^{-7}	3.44×10^{-5}
	150	0.0110	0.0130	1.0×10^{-6}	7.92×10^{-5}
	200	0.0062	0.0159	9.5×10^{-7}	5.97×10^{-5}
	250	0.0040	0.0087	2.3×10^{-6}	2.60×10^{-4}

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-18: Summary of EMS Sensitivity and Mutability Data for HSC-99 in the Presence of a Feeder Layer

Exp	Dose	cl. eff.	n.s. c.e.	s. c.e.	m.f.
D-133 (TK6)	0	0.0253	0.0127	1.4×10^{-6}	1.12×10^{-4}
	100	0.0076	0.0130	6.2×10^{-7}	4.78×10^{-5}
	150	0.0072	0.0080	1.2×10^{-6}	1.45×10^{-4}
	200	0.0047	0.0087	1.6×10^{-6}	1.86×10^{-4}
	250	0.0033	0.0069	2.6×10^{-6}	3.80×10^{-4}

Exp = experiment number; Dose = EMS dose in ug/mL;
cl. eff. = cloning efficiency following EMS treatment
n.s. c.e. = non-selection cloning efficiency following recovery/expression period
s. c.e. = selection cloning efficiency following recovery/expression period
m.f. = mutant frequency

Table AII-19: Welsch Regression Estimates for Control, RS-, RS+ and FA LCLs in the Absence of a Feeder Layer

Cell Line	Regression Equation for Mutant Frequency
DM	$-69.52 + 4.49\ \text{dose} - 0.015\ \text{dose}^2 - 61.34\ \text{exp}\ 1 - 53.8\ \text{exp}\ 2 + 84.72\ \text{exp}\ 3 + 322.2\ \text{exp}\ 4 + 253.15\ \text{exp}\ 5 + 51.72\ \text{exp}\ 6 + 245.63\ \text{exp}\ 7$
HSC-55	$-6767.2 + 93.36\ \text{dose} - 0.23\ \text{dose}^2$
JaKr	$44.15 - 0.29\ \text{dose} + 0.002\ \text{dose}^2 + 23.54\ \text{exp}\ 1 + 32.05\ \text{exp}\ 2$
PeKr	$-201.58 + 3.55\ \text{dose} - 0.012\ \text{dose}^2$
LoGi	$67.61 + 0.31\ \text{dose} + 0.0003\ \text{dose}^2 - 76.05\ \text{exp}\ 1$
TiBu	$102.57 - 0.886\ \text{dose} + 0.004\ \text{dose}^2$
LB-1	$10.67 + 0.15\ \text{dose} - 0.0006\ \text{dose}^2 - 14.59\ \text{exp}\ 1 - 5.68\ \text{exp}\ 2 - 14.59\ \text{exp}\ 3 - 16.24\ \text{exp}\ 4$
R20	$<(-37.41 + 1.43\ \text{dose} - 0.006\ \text{dose}^2 + 88.00\ \text{exp}\ 1 + 72.98\ \text{exp}\ 2)$
HSC-99	$50.89 - 0.82\ \text{dose} + 0.0066\ \text{dose}^2$

Table AII-20: Welsch Regression Estimates for Control, RS-, RS+ and FA LCLs in the Presence of a Feeder Layer

Cell Line	Regression Equation for Mutant Frequency
DM	$502.46 - 3.789\ \text{dose} + 0.016\ \text{dose}^2$
HSC-55	$82.76 - 14.37\ \text{dose} + 0.2525\ \text{dose}^2$
JaKr	$48.85 - 0.083\ \text{dose} + 0.0021\ \text{dose}^2 - 37.61\ \text{exp}\ 1$
PeKr	$-46.78 + 1.61\ \text{dose} - 0.0047\ \text{dose}^2 - 17.02\ \text{exp}\ 1$
LoGi	$223.87 - 4.16\ \text{dose} + 0.036\ \text{dose}^2$
TiBu	$-25.81 + 0.35\ \text{dose} + 0.0022\ \text{dose}^2$
LB-1	$11.19 + 0.61\ \text{dose} - 0.0022\ \text{dose}^2 - 33.87\ \text{exp}\ 1$
R20	$786.4 + 5.27\ \text{dose} - 0.015\ \text{dose}^2 - 984.0\ \text{exp}\ 1$
HSC-99	$111.96 - 1.60\ \text{dose} + 0.011\ \text{dose}^2$