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CELLULAR AND GENETIC CHARACTERIZATION OF UV SENSITIVE CHINESE HAMSTER MUTANTS

M. STEFANINI, C. MONDELLO, E. BOTTA, R. RIBONI and F. NUZZO

Istituto di Genetica Biochimica ed Evoluzionistica del CNR, Pavia, Italy

Summary. - *Results of cellular and genetic characterization of UV sensitive clones (UV^s) isolated from CHO-K1 cell line are reported. The cross-sensitivity to agents inducing a variety of DNA lesions, the induction of chromosome aberrations and of 6-thioguanine and ouabain resistant mutants, the occurrence of methotrexate resistant cells were analyzed in clones showing different degrees of UV sensitivity. Genetic analysis was performed by complementation analysis of hybrids obtained by fusion of our mutants with UV^s cells belonging to the six complementation groups (c.g.) so far identified. Three clones were assigned to c.g. 2, one clone to c.g. 5. Two clones (CHO7PV and CHO4PV), were able to complement each other and showed complementation after fusion with any of the six c.g.; these clones were considered carriers of two new mutations in genes presumably involved in DNA repair.*

Riassunto (Caratterizzazione cellulare e genetica di mutanti UV sensibili isolati da una linea di criceto). - *Si riportano i risultati relativi alla caratterizzazione di mutanti sensibili all'irradiazione con luce UV (UV^s) isolati dalla linea di criceto CHO-K1. In cloni con diversi gradi di sensibilità all'UV è stata analizzata la sensibilità crociata ad agenti che inducono diversi tipi di danno al DNA, l'induzione di aberrazioni cromosomiche e di mutazioni per resistenza alla 6-tioguanina e all'ouabaina; è stata studiata inoltre la frequenza di comparsa del fenotipo resistente al metotressato. L'analisi genetica eseguita confrontando mediante test di complementazione i nostri mutanti con mutanti UV^s appartenenti ai sei gruppi di complementazione (g.c.) finora identificati, ha permesso di attribuire tre cloni al g.c. 2, un clone al g.c. 5 mentre due cloni (CHO7PV e CHO4PV) sono risultati diversi da tutti gli altri e quindi rappresentanti di due nuove mutazioni responsabili di un difetto della riparazione del DNA.*

Introduction

The study of repair processes in man has made considerable headway since the discovery of inherited diseases with altered repair mechanisms. Cells of patients with disorders such as xeroderma pigmentosum (XP), ataxia telangiectasia (AT), Fanconi's anemia (FA) show hypersensitivity to some DNA damaging agents and are defective in functions normally curing the damage induced by UV light, ionizing radiation and bifunctional alkylating agents, respectively [1]. All data obtained so far using the naturally occurring human mutants generally lead to the conclusion that the biochemical mechanisms of DNA repair, and genetic control over these processes in higher organisms are highly complex. In DNA repair deficiency diseases, heterogeneity has been demonstrated by using cell fusion techniques; ten complementation groups have been identified in XP, four in AT and three in FA.

New possibilities to investigate the cellular and molecular aspects of DNA repair in man have been opened up by the availability of mutants isolated *in vitro* from cell lines [2-3]. Established cells do not have a limited life span, are easily handled and therefore offer advantages when extensive selection and cloning procedures are required. Novel aspects of repair processes have been revealed by the cellular characterization of the laboratory-derived mutants that proved also to be the successful tool for the mapping and the cloning of human DNA repair genes [4-6].

We were interested in the isolation of Chinese hamster cell lines phenotypically resembling the naturally occurring mutant XP. For this purpose, we have worked out a relatively simple procedure that allows us to isolate a panel of UV sensitive (UV^s) clones from the CHO-K1 cell line; cellular and genetic studies performed in several of these mutants are presented. Part of these results have been already published.

Isolation of *in vitro* UV^s mutants

The approach followed to isolate UV^s mutants employed conventional mutagenesis, enrichment for UV^s mutants, identification and isolation of the repair-deficient clones. The procedure has been already extensively described [7-8]; briefly, the cells were maintained in growth medium for 16 h with 3 mM ethyl methane sulfonate to increase the mutation frequency and subcultured for 8 days to allow complete recovery from mutagen treatment and mutation expression. The mutagenized population was enriched in repair defective mutants by killing cells with normal UV sensitivity. To this purpose, the cells were stimulated to perform repair synthesis by irradiation with a low dose of UV light and then incubated in the presence of bromodeoxyuridine (BrdU), a thymidine analogue, or ^{3}H-thymidine (^{3}H-TdR). Cells proficient in repair synthesis incorporate into their DNA the potentially harmful substance; the subsequent irradiation with visible light (after BrdU incorporation) or the radioactive decay of tritium in liquid nitrogen (after ^{3}H-TdR incorporation) leads to the preferential survival of mutants unable to perform repair synthesis.

The surviving colonies were then replica-plated on filter paper; the clones in the master plate were screened for UV-sensitivity by measuring their ability to perform UV-induced DNA repair synthesis (UDS) or their growth capability after UV irradiation; the colonies unable to perform UDS or to increase in size after UV were considered putative UV^s clones. The corresponding colonies were isolated from the replica, grown up and retested for UV sensitivity.

Using this procedure, we isolated a number of clones in seven independent experiments and 25 of them appeared to be characterized by reduced colony forming ability after UV light. Their UV sensitivity is not extreme, as expected as a consequence of the approach followed for their isolation. In fact, during the enrichment procedure the cells are exposed to UV light that kills the most sensitive ones. A great heterogeneity in the level of UV sensitivity was observed among different clones. Survival curve parameters (D_{37}, D_q, D_0) and UDS after UV in a representative sample of clones are reported in Table 1. The low levels of UDS together with the reduced survival after UV make these Chinese hamster mutants analogs of XP cells.

Cellular analysis

In several mutants we analyzed the chromosomal constitution, the growth properties and the stability of the mutation conferring UV sensitivity. Compared to parental cells, the mutants did not present different growth capabilities, or specific alterations in karyotype.

Table 1. - *Sensivity to UV in wild type and UV^S CHO cell lines*

	UDS (*)	D_{37}	D_0	Dq
CHO-K1	100	11.0	3.8	7.1
CHO4PV	59	7.5	2.7	4.7
CHO7PV	30	7.0	2.5	4.5
CHO10PV	72	9.1	2.5	6.5
CHO23PV	47	8.2	2.8	5.3
CHO30PV	39	5.2	2.3	2.9
CHO40PV	36	6.4	1.5	4.8
CHO50PV	34	5.6	1.4	4.8
CHO60PV	37	6.8	1.8	4.8
HO423PV	54	5.1	2.1	3.0

(*) % of UDS in wild type CHO-K1 cells. UDS analysis was performed on autoradiographic preparations.
The survival parameters D_{37}, D_0, D_q were obtained from the linear regression curves (R) calculated by the least square method using the values in the exponential part of the curve. D_{37} is the UV dose required to reduce survival to 37%; D_q is the dose at which survival is 100% on the regression curve. D_0 is the dose required to reduce survival to 37% on the curve parallel to R and intercepting the ordinate at the value 1.

Over a period of nine months in culture all the clones showed no increase in the UDS level or in survival after UV.

To obtain data on the relationship between DNA damage, repair, cytotoxicity and mutability, clones showing different UV sensitivity were analyzed for UV-induced chromosome aberrations and mutation. The effect of UV light was evaluated in mitotic cells of seven UV^s clones at different times after irradiation (17, 41, 65 h). The mitotic index values decreased as a function of UV dose similarly in the parental cells and in four UV^s clones (CHO7PV, CHO10PV, CHO30PV, CHO211PV); the reduction was more marked in the other three UV^s mutants (Table 2). The mean number of chromosomes did not show any marked variation with increasing UV doses both in UV^s and parental cells (Table 3) indicating that in cells capable of undergoing mitosis, UV light does not induce non-disjunction events, which are the main cause of chromosome loss.

A clear increase in chromosome anomalies is observed after UV both in wild-type and UV^s cells. The lesions consist mainly of chromatidic breaks and rearrangements (triradial, quadriradial or more complex figures) (Fig. 1). Chromosomal rearrangements (such as dicentrics and rings) are not observed in cultures harvested 17 h after irradiation and are present with low frequency at longer incubation times.

Heterogeneity in the clastogenic effect of UV light was observed among the UV^s clones (Table 4). Frequencies of aberrant mitoses and breakage rate similar to those observed in parental cells are present in CHO211PV (over the entire range of doses tested) and in CHO10PV (up to 12 J/m^2) cell lines. In four other UV^s cell lines the values were even lower than those in parental cells. In CHO43RO, which is the most sensitive

Table 2. - *Mitotic indices (% of the value in unirradiated cultures) in wild type and UV^S CHO cell lines*

Cell line	UV dose (J/m^2) 6	12	18	24
CHO-K1	49.9	22.2	13.6	4.4
CHO7PV	53.6	19.3	11.9	3.4
CHO10PV	60.8	20.1	6.7	3.3
CHO23PV	15.3	7.3	5.0	n.t.
CHO30PV	67.4	63.1	13.9	n.t.
CHO43RO	21.5	8.6	4.4	4.2
CHO211PV	47.1	17.0	4.6	2.6
CHO423PV	26.1	10.6	10.3	5.2

n.t. = not tested
Cells were harvested 17 h after UV irradiation and maintained in the presence of Colcemid for the last 2 h

Table 3. - *Mean number of chromosomes in wild type and UV^S CHO cell lines 17 h after UV irradiation*

Cell line	UV dose (J/m^2) 0	6	12	18	24
CHO-K1	19.7	19.4	19.4	19.2	19.5
CHO7PV	18.1	18.0	17.9	18.1	18.3 *
CHO10PV	19.7	19.7	19.4	19.5 *	19.1 *
CHO23PV	19.4	19.2	18.9	18.9	n.t.
CHO30PV	18.7	19.2	18.7	19.0	n.t.
CHO43RO	19.0	19.7 *	19.4 *	19.2 *	18.4 *
CHO211PV	19.7	19.4	18.9	18.9 *	19.2 *
CHO423PV	18.9	19.0	18.7	19.3 *	18.8 *

n.t. = not tested
* analysis was performed on less than 100 mitoses

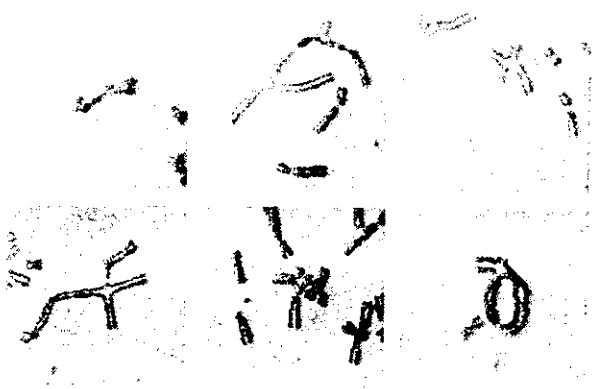

Fig. 1. - Examples of chromosome aberrations induced by UV irradiation in wild-type and UV sensitive CHO cell lines.

clone among the mutants analyzed, the values were higher than those in parental cells. In conclusion the clastogenic effect of UV light was more marked in UV^s cells showing high cellular UV hypersensitivity.

Similar results were obtained by analyzing the UV-induced frequency of mutants at the loci conferring resistance to 6-thioguanine and ouabain. The study performed on three UV^s clones indicated that the response to UV-mutagenesis at the two markers was enhanced only in the most sensitive one [9].

The frequency of UV-induced methotrexate resistant (MTX^R) cells was analyzed in wild-type and CHO43RO cells [10]. The aim of this investigation was to verify whether the mutational events leading to MTX resistance are related to the damage induced in the DNA molecule and to the repair processes.

Resistance to MTX in mammalian cells can occur as a result of: i) alteration of the dihydropholate reductase (DHFR), which affects the specificity of the drug; ii) mutations in the membrane transport system that prevent the drug from reaching the target enzyme; iii) DHFR gene amplification, which leads to an increased cellular level of the enzyme [11]. Evidence has been obtained that in the single step selection the frequency of MTX^R cells from mouse and hamster cell lines is increased by pretreatment with agents that interfere with DNA metabolism, such as MTX, UV-rays, hydroxyurea [12].

In our experiments, the MTX^R frequency showed a positive correlation with the UV dose and was higher in the UV^s than in the parental CHO cells. Four and ten MTX^R clones were isolated from wild-type and CHO43RO cell lines respectively; these clones, were characterized for frequency of reversion, chromosome constitution, amplification of the DHFR gene.

In all but one MTX^R clone the resistance marker was still present after two months' culture in the absence of the drug.

Cytogenetic analysis on G-banded chromosomes showed the presence of numerical and structural aberrations involving chromosome No 2 in two and seven MTX^R clones isolated from CHO-K1 and CHO43RO cell lines respectively. The most frequent finding was the trisomic condition for the long arm of this chromosome on which the DHFR gene is located. In two clones the occurrence of amplification of the DHFR gene was revealed by nucleic acid hybridization techniques.

The overall data indicate that all the three classes of mutations underlying the MTX^R phenotype increase with UV exposure. Furthermore the higher frequency of mutants in repair-deficient cells compared to wild-type cells suggests that the persistence of damage in the DNA molecule promotes amplification processes.

Eight mutants were characterized for sensitivity to DNA damaging agents by measuring the inhibition of colony-forming ability after exposure to increasing doses of two monofunctional alkylating agents (ethyl methanesulfonate, EMS; methyl methanesulfonate, MMS), two bifunctional alkylating agents (mitomycin C, MMC; 1,2-chloroethyl-3-cyclohexyl-1-nitrosourea, CCNU), and hydrogen peroxide (H_2O_2). This study clearly indicated that most of the UV^s clones show hypersensitivity to agents inducing a variety of damages (Table 5). If a single mutation in each clone is hypo-

Table 4. - *Breakage rate and frequency of mitoses with breaks and rearrangements in wild type and UV^S CHO cell lines 17 and 41 h after UV irradiation*

UV dose (J/m^2)	Abnormal mitoses (%) 6	12	18	24	Mean number of breaks/cell 6	12	18	24
17 h								
CHO-K1	12.3	22.7	30.7	42.7 *	0.29	0.66	0.80	1.16 *
CHO7PV	7.0	12.0	16.0	19.0 *	0.14	0.33	0.43	0.33 *
CHO10PV	12.5	23.5	22.8	24.0 *	0.23	0.43	0.54	0.36 *
CHO23PV	5.0	13.0	15.0	n.t.	0.07	0.20	0.25	n.t.
CHO30PV	9.0	15.0	22.0	n.t.	0.19	0.34	0.45	n.t.
CHO43RO	21.9	48.0 *	n.t.	n.t.	0.68	1.08 *	n.t.	n.t.
CHO211PV	16.0	24.0	30.4	40.0	0.36	0.55	0.77	0.93
41 h								
CHO-K1	7.5	24.5	36.1	35.2 *	0.17	0.54	1.03	1.20 *
CHO7PV	3.0	11.0	23.9	50.0 *	0.06	0.28	0.82	1.10 *
CHO43RO	25.0 *	33.3 *	n.t.	n.t.	0.78 *	0.78 *	n.t.	n.t.
CHO211PV	5.0	16.0	25.0	35.0	0.08	0.48	0.58	0.84
CHO423PV	11.0	19.4	27.6	31.4 *	0.20	0.64	0.64	0.97 *

* analysis was performed on less than 100 mitoses

In unirradiated cultures the frequency of abnormal mitoses was less than 1.1% and the mean number of breaks/cell less than 0.03

n.t. = not tested

Table 5. - *Sensitivity to DNA damaging agents in UV^S clones*

	MMS	EMS	MMC	CCNU	H_2O_2
CHO4PV	+++	++	+++	+++	n.t.
CHO7PV	+	+	+++	+	++
CHO10PV	+	++	=	++	++
CHO23PV	++	+	=	++	n.t.
CHO30PV	+	=	+	+	=
CHO43RO	+	=	+++	+++	=
CHO211PV	=	++	n.t.	=	n.t.
CHO423PV	=	=	+	=	=

\+ = D_{37} values reduced to 90-70%

++ = D_{37} values reduced to 70-50%

+++ = D_{37} values less than 50% of the corresponding D_{37} values in wild-type cells

n.t. = not tested

Table 6. - *Genetic analysis of the DNA repair defects in UV^S Chinese hamster clones*

Fusion parental cells	UV4 c.g. 1	UV20 c.g. 2	UV24 c.g. 3	UV41 c.g. 4	UV135 c.g. 5	UV161 c.g. 6	CHO7PV
CHO43RO	+	-	+	+	+	+	+
CHO423PV	+	-	+	+	+	+	+
CHO30PV	+	-	+	+	+	+	+
CHO50PV	+	+	+	+	-	+	+
CHO7PV	+	+	+	+	+	+	-
CHO4PV	+	+	+	+	+	+	+

Presence (+) or absence (-) of complementation in hybrids

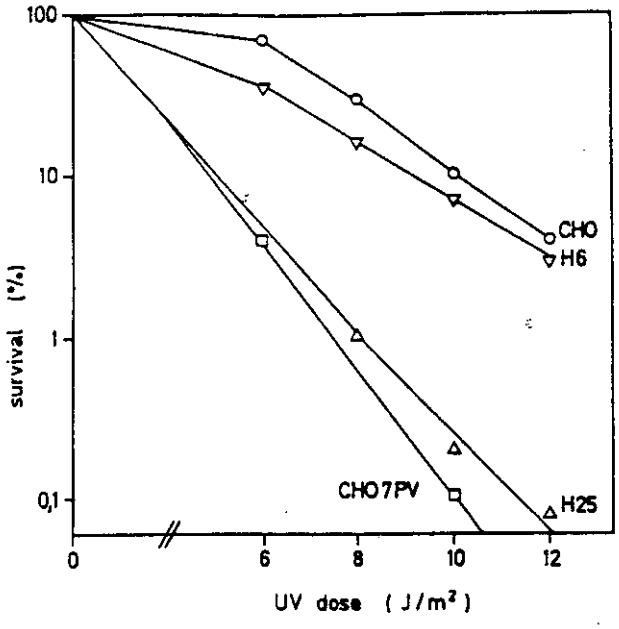

Fig. 2. - Colony-forming ability after UV irradiation in the CHO-K1 cell line, in the UV^s CHO7PV clone and in two hybrids (H6, H25) obtained after fusion of CHO7PV cells with human lymphocytes.

thesized, this finding indicates an overlap in the DNA repair pathways involved in the restoration of different lesions.

Different sensitivity to EMS and MMS in the same clone might be correlated to a defect in the removal of ethylated or methylated bases or to differences in the capability to repair site-specific lesions. Different sensitivity to MMC and CCNU in the same clone may be due to incomplete overlapping of the damage induced by the two bifunctional alkylating agents or to different efficiencies in the formation of monoadducts and cross-links. Increased sensitivity to H_2O_2 (as observed in CHO7PV and CHO10PV clones) indicates that the oxygen radicals are not eliminated normally by antioxygenic factors (enzymes such as catalase, peroxidases, superoxide dismutases and low molecular weight free-radical scavengers and antioxidants).

Genetic analysis

Genetic analysis of the DNA repair defect was performed in six mutants as previously described [13] by measuring survival after UV irradiation in hybrids obtained after fusion of mutant cells with wild-type or UV^s cells representative of the six Chinese hamster complementation groups (c.g.) so far identified [14-15]. Briefly, parental cells carrying aminopterine and ouabain resistance, respectively, are fused in suspension and seeded in dishes; 24 h later cells are UV irradiated and incubated at 37 °C in selective medium for 8 days at which time surviving clones are scored.

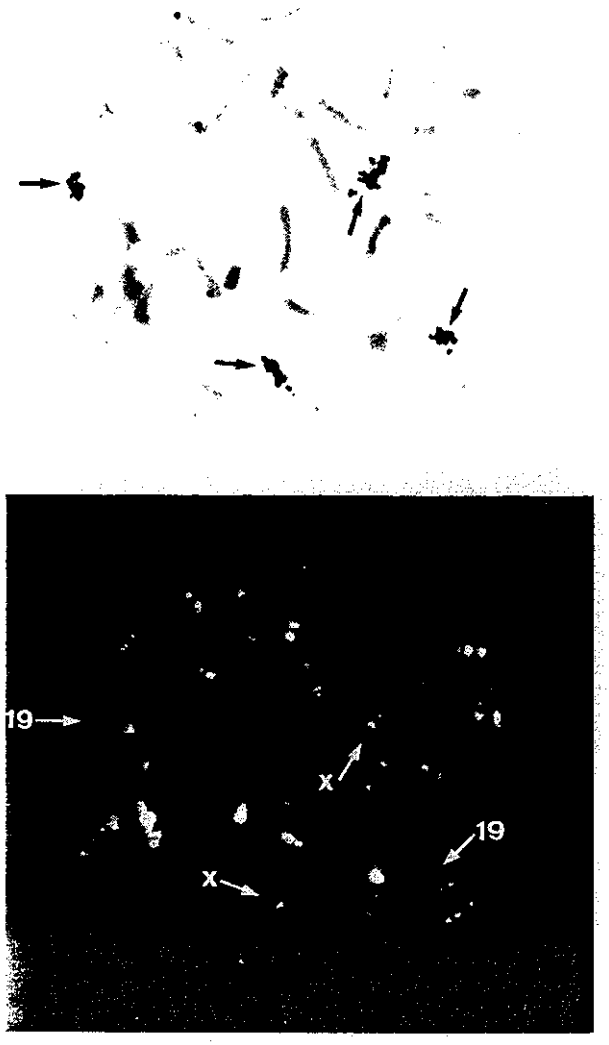

Fig. 3. - Metaphase of a human CHO7PV hybrid. *In situ* hybridization with human genomic DNA (upper part); G-banding (lower part). The arrows point to the human chromosomes.

After fusion with wild-type cells, the six UV^s mutants showed a marked increase in the survival levels after UV irradiation indicating that the mutation in all the clones behaves as a recessive character. Complementation studies in hybrids between our UV^s clones and mutants representative of the six c.g. are reported in Table 6.

Three clones (CHO43RO, CHO423PV, CHO30PV) appeared to belong to c.g. 2 which is the most represented c.g. since half out of about 150 UV^s Chinese hamster mutants analyzed by Thompson and Carrano [16] fit into this class. It is worth noticing that our clones, despite their homology at the genetic level, show heterogeneous phenotype (different degrees of mutagen sensitivity, UV-induced repair synthesis, chromosomal fragility) suggesting that defects in the same genetic locus may result in different degrees of phenotypic alterations [13].

The fourth mutant analyzed (CHO50PV) did not show complementation after fusion with UV135 cells, which are representative of the c.g. 5. Mutants belonging to c.g. 5 seem to occur frequently in the mouse lymphoma L5178Y cell line: three among eight independent isolates have been classified in this c.g. [17]. In contrast CHO50PV is the second CHO UV^s mutant fitting into c.g. 5, since UV135 was the only representative of this class among clones isolated from CHO-AA8 cell line by Thompson and Carrano [16].

Finally the last two clones analyzed (CHO4PV and CHO7PV), were able to complement each other and showed complementation after fusion with cells belonging to any of the six Chinese hamster c.g.: therefore they represent two new complementation groups.

The availability of DNA-repair mutants carrying mutations other than those so far identified offers the opportunity to study new functions involved in the restoration of DNA damage in mammalian cells.

Attempts to isolate the gene that corrects the defect in CHO7PV cells have been so far unsuccessful since the low mutagen hypersensitivity of CHO7PV does not provide an efficient selection system for cells transfected with human DNA.

Efforts toward mapping on human chromosomes the genes correcting the repair defect in CHO7PV and CHO4PV cells are under way. Hybrids between these mutants and human lymphocytes have been obtained and their characterization for human chromosome content and UV sensitivity is in progress. UV sensitivity is analyzed by measuring survival after UV irradiation (Fig. 2); human chromosomes are recognized by *in situ* hybridization of human genomic DNA to metaphases and identified by G-banding (Fig. 3).

A last aspect concerning the genetic homology between the mutations conferring UV sensitivity in the CHO7PV and CHO4PV mutants and human XP cells is under investigation by complementation studies.

Note

At the UCLA meeting on "Mechanisms and consequences of DNA damage processing" in Taos (January 1988) CHO complementation group numbering was changed to comply with ERCC-gene numbering: the group 2 is now group 1 and vice versa.

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