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Constitutive over-production of DNA-damage recognition proteins and acquired UV resistance in prolonged culture of F9 teratocarcinoma stem cells

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An ultraviolet (UV)-resistant F9 variant cell line, termed F9Vc, was established from a prolonged culture of murine F9 embryonic stem cells. A 6-fold UV resistance was detected in F9V2 cells compared to the F9 parental cells, as determined by ID_{50} (36 J/m^2 vs. 6 J/m^2), the UV dose causing 50% growth inhibition. Using a DNA mobility-shift assay, a nuclear protein (termed UVDRP) that preferentially binds to UV-damaged DNA was detected in F9 and F9Vc cell extracts. The UVDRP in F9Vc cells was present at a 7-fold higher concentration than that of F9 cells. Interestingly, the F9 UVDRP was transiently induced following cellular differentiation by retinoic acid (RA)/cAMP, with optimum induction (15-fold) at 6 days. Although constitutively over-produced, UVDRP also remained inducible in F9Vc cells in response to RA/cAMP. Indirect DNA repair measurement by host cell reactivation of UV-damaged plasmid DNA demonstrated that F9Vc cells exhibited a slight increase or a similarity in repair ability compared to the F9 cells. Parallel experiments using the repair-defective xeroderma pigmentosum (XP) group A fibroblasts and the normal VA13 fibroblasts also indicated that over-production of UVDRP binding activity was associated with enhanced DNA repair and UV resistance. The findings indicate that prolonged culture of F9 cells can establish a condition sufficient to cause constitutive over-production of UVDRP binding activity and UV resistance. The results also suggest that the RA/cAMP-inducible UVDRP in F9 stem cells may be important for the sensitivity or resistance of the cells to UV damage.

DNA damage recognition; Retinoic acid; UV resistance

1. INTRODUCTION

Ultraviolet (UV) radiation causes cyclobutane-type pyrimidine dimers, (6–4) photoproducts, as well as other DNA base adducts in cells. In response to this naturally occurring DNA-damaging agent, a variety of systems have evolved for the repair of damaged DNA [1]. Recently we and others have found an increased UV-damaged DNA recognition protein (UVDRP) binding activity in human cells resistant to UV [2,3]. This is supported by the findings that nuclear extracts isolated from mammalian and yeast DNA-repair mutants fail to interact with damaged DNA [4–6]. It has been reported that some cells resistant to cisplatin, a potent chemotherapeutic agent, expressed enhanced plasmid reactivation and induced binding activity for cisplatin DNA adducts [2,7–9]. The removal of the major cisplatin adducts involves nucleotide excision repair in bacterial [10–12] and in mammalian cells [13,14]. These results argued that the repair process of the cisplatin adduct is probably the same, at least in part, as that of 'UV-type' excision repair. However, failure to detect an increase in cisplatin–DNA recognition activity in cisplatin-resistant cells has been reported by others [15], suggesting complexities in the regulation of damage recognition activity in cells. Although the exact similarity or dissimilarity between cisplatin- and UVDRP is not clear, inducible UVDRP has been demonstrated in primate cells by pre-treatment of cells with UV [16]. However, the regulation of UVDRP binding activity is not clear.

In this study, I report the cross-resistance to UV radiation of a variant cell line derived from a prolonged culture of murine F9 teratocarcinoma stem cells. This F9 variant cell line also exhibits over-production of a nuclear protein that specifically interacts with UV-damaged DNA. Interestingly, the UVDRP binding activity is inducible in differentiating F9 and the variant cells by retinoic acid (RA)/cAMP. The low constitutive level of UVDRP and an inefficient host reactivation of UV-damaged plasmid DNA in xeroderma pigmentosum (XP) cells have important implications in that UVDRP binding activity may play a role in cellular sensitivity or resistance to UV radiation.

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Abbreviations: CAT, chloramphenicol acetyltransferase; DRP, damage-recognition protein; ID_{50}, dose resulting in 50% inhibition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RA, retinoic acid; UV, ultraviolet radiation; XP, xeroderma pigmentosum.

2. MATERIALS AND METHODS

2.1 *Cell lines and culture conditions*

Murine F9 embryonic adenocarcinoma stem cells, the F9 variant cells (F9Vc), and human VA-13 and XP fibroblasts, were maintained according to the supplier's specifications (American Type Culture Collection). F9Vc variant cells were derived from a prolonged culture (10–15 passages) of F9 cells. XP fibroblasts (XP12RO, SV40 transformed xeroderma pigmentosum complementation group A) and a normal human fibroblast WI38-derived VA13 cell line, were grown in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) containing 10% (v/v) heat-inactivated fetal calf serum, and incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air. As for the human cells, F9 cells were cultured on gelatin-coated (0.1%) tissue culture dishes in Dulbecco's modified Eagle's medium.

2.2. *Determination of cytotoxicity*

Inhibition of cell growth was assayed by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as previously described [17]. The fold sensitivity of cells to UV was defined as the ratio of the ID_{50}, the UV dose causing 50% growth inhibition, between the tested cell line and the reference line.

2.3. *Induction of differentiation*

To induce differentiation, F9 and F9Vc cells were treated with 0.1 μM *all-trans*-retinoic acid (RA) and 1 mM dibutyryl cAMP (Sigma Chemical Co., St. Louis, MO). Establishment of a differentiated phenotype was evident from morphological changes in response to RA/cAMP exposure. Biochemical differentiation was verified by analyzing cell cultures for expression of plasminogen activator [18].

2.4. *DNA transfection and CAT assays*

pRSVcat DNA was introduced into cells by the electroporation technique [19] as described by the manufacturers' instructions (GenePulser; Bio-Rad). Cells were seeded at 3×10^6 cells per 100-mm plate 1 day before electroporation. 1 ml of cell suspensions, in HEPES buffer, was added to a sterile cuvette containing 20 μg pRSVcat and 10 μg pSVβ (Clonetech) plasmids, gently mixed, and subjected to electroporation. Conditions with 1,000 μF capacity and 200 V were typically used. The following day the cells were fed with fresh media and incubated for another 48 h in the presence or absence of RA/cAMP. Cells were then harvested for CAT assay as previously described [20].

The CAT assay reaction was incubated at 37°C for 1 h, followed by development on silica thin-layer chromatography (TLC) plates (Macherey-Nagel, Germany). The β-galactosidase activity of the same preparation for CAT assay was also analyzed as an internal control [18]. After autoradiography, density on the X-ray film corresponding to the modified chloramphenicol was quantitated by scanning densitometry (Hoefer GS300). After normalization to the β-galactosidase activity, the CAT activity was calculated as % of chloramphenicol converted into acetylated derivatives. The ID_{50} for plasmid reactivation was defined as the UV dose which resulted in 50% inhibition of the CAT activity.

2.5. *DNA probes and DNA mobility-shift assay*

DNA probes were prepared as described [21]. The DNA fragment, f130, was originally isolated from plasmid pSVT [22] and inserted into vector pBS(+) (Stratagene). The *Hin*dIII–*Eco*RI f130 fragment containing a 17 bp dA/dT-rich region is a potential target for UV modification. *Hin*dIII and *Eco*RI-generated f130 fragments were [^{32}P]dCTP-labeled (3×10^4 cpm/ng DNA) using Klenow DNA polymerase, and purified in spin columns by standard methods [18]. The f130 DNA at a concentration of 100 μg/ml was irradiated with UV germicidal lamps as previously described [23]. DNA was irradiated at a dose rate of 25 J/m^2/s from a VL-100C UV irradiation unit (Vilbert Lourmat, France). The dose rate was measured by a VLX-254 radiometer (Vilbert Lourmat, France). For some cases, a double-stranded synthetic oligonucleotide sequence (Sp1 site), 5'-GATCGATCGGGGCGGGGCGATC-3' (Stratagene Cloning Systems, La Jolla, CA), was used as a control probe.

Crude nuclear and cytosolic extracts were prepared according to Dignam et al. [24]. The protein concentration was measured via the Bradford assay using the Bio-Rad dye reagent [25], and visualized by SDS-PAGE [26]. Protein–DNA binding using 0.3 ng DNA probe, unless otherwise specified, was conducted in 15 μl of buffer containing 12% glycerol, 12 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 4 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 300 μg/ml BSA at 25°C for 30 min as described [27]. The reaction mixtures were then subjected to (4%) polyacrylamide gel-electrophoresis under low ionic strength (6.7 mM Tris-HCl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA) at 25°C and 15 mA constant current. The resolved gel was dried and exposed to Kodak XAR-5 X-ray film at −70°C with an intensifying screen. The intensity of the shifted DNA bands was determined by scanning densitometry. The relative binding was determined by $b/b + f$, where b is bound probe and f is free probe.

3. RESULTS

3.1. *Resistance of F9Vc cells to UV radiation*

UV sensitivity of F9Vc and F9 cells was compared using growth inhibition assays (Fig. 1: for comparison, VA13 and XP group A cells are also shown). Cells were exposed to 0, 5, 10, 20, 40, or 80 J/m^2 of UV, and analyzed after 4 days of incubation. Relative growth inhibition (R.G.I.) vs. UV dose indicates that F9 parental cells are extremely sensitive to UV. The ID_{50} (UV dose causing 50% growth inhibition) of F9 is comparable to that of XP cells, known to be defective in DNA

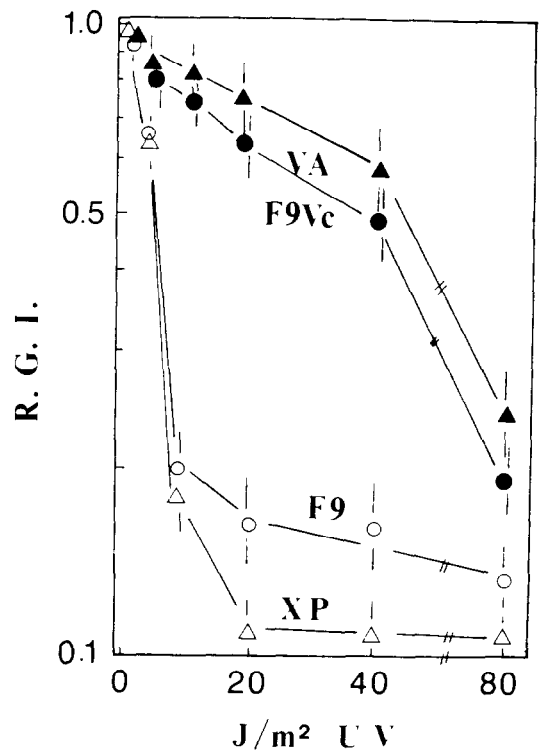

Fig. 1. Relative growth inhibition (R.G.I.) of F9, F9Vc, VA, and XP cells. Points with error bars (S.D.) show the mean of 5 separate experiments.

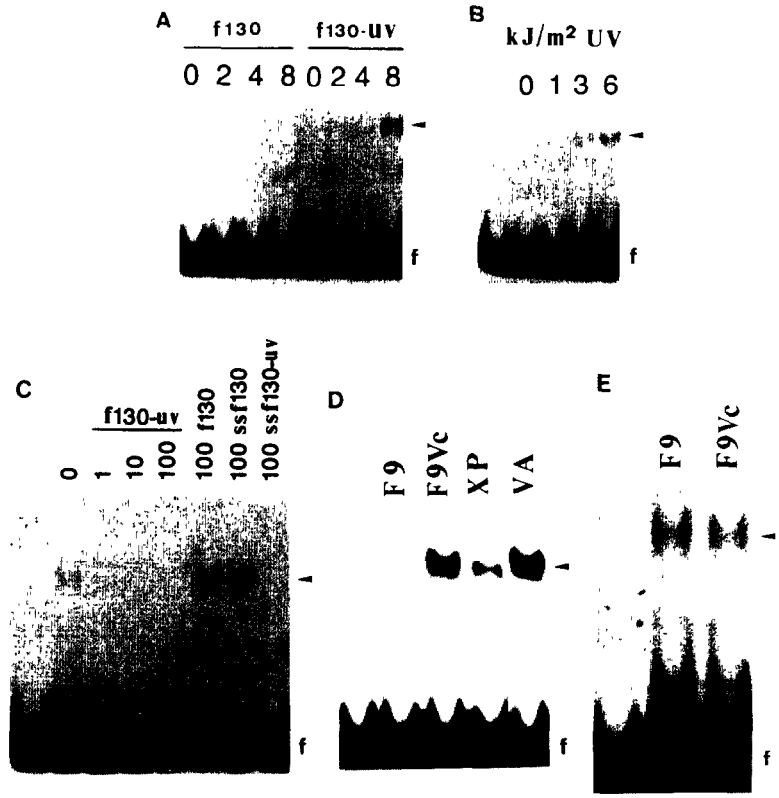

Fig. 2. UVDRP binding activity in F9 and F9Vc cells. (A) Protein-dependent UVDRP binding activity. 0, 2, 4, or 8 μg of nuclear extracts prepared from F9 cells was incubated with unirradiated f130, or 6,000 J/m^2 UV-irradiated f130 (f130-uv). (B) UV dose-dependent UVDRP binding activity The F9 nuclear extracts (8 μg) were incubated with f130 which had been irradiated with 0, 1, 3 or 6 kJ/m^2 UV. 6 J/m^2 UV-irradiated f130 probe without nuclear extracts (left lane) is shown as a control. (C) Specificity of the UVDRP binding activity. Probe (0.3 ng) was incubated with 8 μg of F9 nuclear extracts in the presence of the indicated molar excess of specific competitor, f130-uv, or non-specific competitor including unirradiated, f130, single-stranded f130 (ssf130), or irradiated single-stranded f130 (ssf130-uv). (D) Comparison of UVDRP binding activity in F9, F9Vc, VA and XP cells. 8 μg (F9 and F9Vc) or 2 μg (XP and VA) of nuclear extracts was studied. (E) Sp1 binding activity in F9 and F9Vc cells. Probe f130-uv (C,D) or Sp1 (E) alone is shown in the left lane as a control. Arrowhead indicates bound probe; f indicates unbound free probe.

repair. In contrast, the F9Vc and VA cells exhibited a greater resistance to UV. There is a 6- to 7-fold resistance of F9Vc and VA cells compared to the F9 and XP cells, calculated from the ID_{50} ratio of the F9Vc and VA cells (36–43 J/m^2) to that of the F9 and XP cells (6 J/m^2) (also see Table I).

Table I

Comparison of parameters in F9 and F9Vc cells

	F9	F9Vc	F9Vc/F9
Growth inhibition (ID_{50}, J/m^2)	6	36	6
UVDRP abundance[a] (molecules per megabase)	0.004	0.028	7
Plasmid reaction (ID_{50}, J/m^2)	200	210	1.05

[a] The relative levels of binding activity from 8 μg of nuclear extracts and 6,000 J/m^2 UV-irradiated DNA probe were determined by $b/b+f$ of three experiments, where b is bound probe and f is free probe.

3.2. *Detection of a UVDRP binding activity in F9 and F9Vc cells*

Under the standard DNA-binding conditions, a UVDRP was detected in F9 and F9Vc cells (Fig. 2). F9 nuclear extracts (0, 2, 4, or 8 μg) were incubated with unirradiated f130 or 6,000 J/m^2 UV-irradiated f130-uv (Fig. 2A). A UV-damage specific binding activity (indicated with an arrowhead; also see below) was detected by the f130-uv probe, but not by f130. UVDRP binding roughly increased with the amount of nuclear extracts. In any case, a majority of the UVDRP binding activity appeared in the nuclear fraction: only slight or no UVDRP was present in the cytosol (data not shown). To test whether the UVDRP binding is proportional to the level of UV-DNA adducts, 0.3 ng of f130 was incubated with 8 μg of F9 nuclear extracts after it had been irradiated with 0, 1, 3, or 6 kJ/m^2 of UV (Fig. 2B). Scanning densitometry analysis indicated a rough correlation between the UVDRP binding and the level of UV-DNA adducts. Probe alone (left lane of Fig. 2B) is shown as a control. Specificity of the UVDRP binding

was investigated using a competition binding assay (Fig. 2C). The f130-uv (0.3 ng) probe was incubated alone (left lane), or with 8 μg of F9 nuclear extracts in the absence or presence of a molar excess of competitors (1-, 10-, or 100-fold, indicated on the top of the figure). The competitors included f130-uv, unirradiated f130, single-stranded f130 (ssf130), or single-stranded f130-uv (ssf130-uv). A majority of the binding activities was inhibited by f130-uv, for example, binding was inhibited by more than 50% when f130-uv competitors were increased by 10-fold. In contrast, unirradiated f130 or single-stranded f130 did not show significant inhibition of UVDRP binding. Unexpectedly, UV-irradiated single-stranded f130 also partially inhibited UVDRP binding. Binding reaction without nuclear extracts (left lane) is shown as a control. In our experience, there is a ~15% deviation in routinely performed DNA-binding assays. The same patterns of UVDRP binding and competition were detected in F9Vc cells (data not shown).

3.3. *Over-production of UVDRP binding activity in F9Vc cells*

Nuclear extracts of F9 (8 μg), F9Vc (8 μg), XP (2 μg) or VA (2 μg) were compared for UVDRP binding (Fig. 2D). The UVDRP binding activity was 7-fold higher in F9Vc cells than in F9 cells. The abundance of the F9 and F9Vc UVDRP was approximately 0.004 and 0.028 molecules per megabase, respectively, if 1:1 binding stoichiometry is assumed. There was also a 7-fold difference in the UVDRP binding activity between XP and VA cells. This is not due to differential protein loading because a comparable binding activity for Sp1 was detected in F9 and F9Vc cells (Fig. 2E). Sp1 is a transcription factor which binds to the DNA sequence 5′-GGGGCGGGGC-3′ [28]. In addition, the amount of protein in each extract was the same, based on SDS-PAGE analysis as well as colorimetric (Bradford) assay. Thus, UVDRP binding activity is constitutively overproduced in F9Vc cells.

3.4. *Induction of UVDRP binding activity in F9 and F9Vc cells by RA/cAMP*

We have previously demonstrated RA/cAMP-mediated induction of UVDRP in F9 cells [29]. To investigate whether the regulation of F9Vc UVDRP is altered, 8 μg of nuclear extracts from F9 and F9Vc cells following 2, 4, 6, or 8 days of induction by RA/cAMP (see section 2) were analyzed. The fold induction was calculated from the ratio of the UVDRP binding activity of induced cells divided by that of uninduced cells (Fig. 3). The binding activity increased with the days of RA/cAMP treatment. Optimal UVDRP binding (15-fold) was detected in both F9 and F9Vc cells at day 6, followed by a rapid decrease at day 8. Thus, UVDRP binding activity can be elicited by treating cells with the differentiating agent, RA/cAMP, in F9 parental and the variant cells. It should be noted that under this induction, 70–80% of the cells appeared to be of a differentiated parietal endoderm phenotype. The data indicate that inducibility of UVDRP in F9Vc cells by RA/cAMP is as normal as F9 cells.

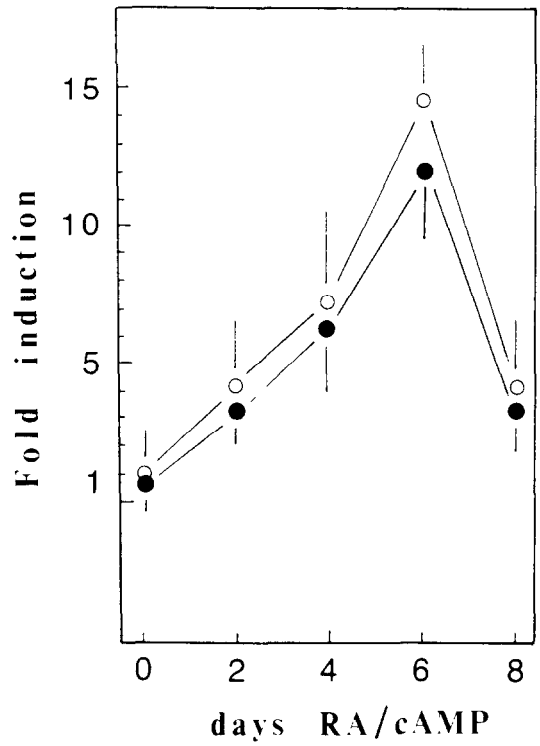

Fig. 3. Induction of UVDRP binding activity in F9 and F9Vc cells by RA/cAMP 8 μg of nuclear extracts isolated from F9 (○) or F9Vc (●) cells induced with the indicated days of RA/cAMP, were incubated with the 6,000 J/m² UV-irradiated probe, f130-UV. Fold induction of UVDRP binding activity with error bars is shown as a function of days of RA/cAMP treatment.

3.5. *Host cell reactivation of damaged plasmid DNA in F9 and F9Vc cells*

Based on studies of cellular sensitivity to UV, one may speculate that F9Vc cells have acquired enhanced DNA repair. To test this, the CAT plasmid carrying DNA with various amounts of UV damage, was transiently expressed in F9, F9Vc, XP and VA cells (Fig. 4). pRSVcat (20 μg) irradiated with 0, 0.25, 1, 2, or 3 kJ/m² of UV was introduced together with 10 μg of pSVβ into cells 60 h before the CAT and β-galactosidase activity assays. The % CAT activity of 3 determinations was quantitated using scanning densitometry. After normalization to the internal control β-galactosidase activity, the relative CAT activity (R.C.A.) was calculated (see section 2). The data indicate that CAT activity decreased with the level of UV damage in all of the cell lines. The transfected F9 (open circle), F9Vc (filled circle) and XP (open triangle) cells showed a much lower CAT activity expressed from UV-irradiated plasmid DNA than VA cells. For example, approximately 50%

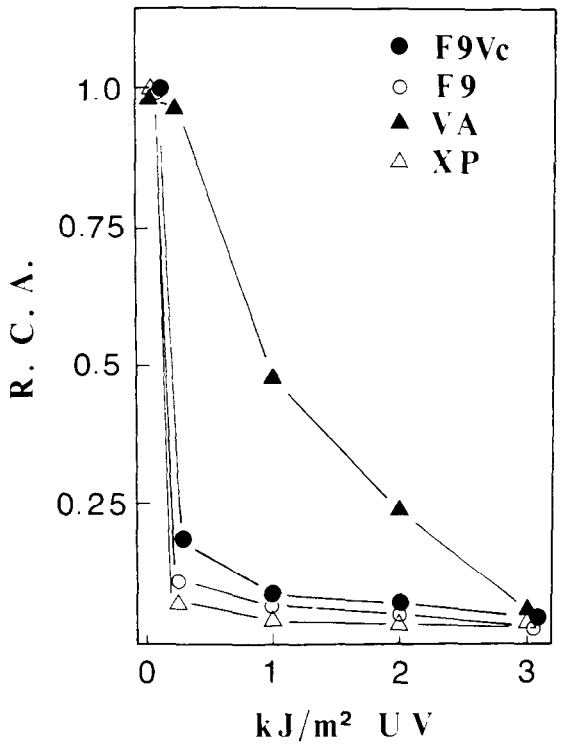

Fig. 4. Host cell reactivation of UV-irradiated pRSVcat plasmid in F9, F9Vc, VA, and XP cells. After normalization to the internal control β-galactosidase activity, relative CAT activity (R.C.A.) is shown as a function of UV dose. The estimated error due to uncertainties in plasmid transfection and the CAT assay was 15–20%, as compiled from 3 experiments.

of the CAT activity was reduced by 0.25 kJ/m^2 in these cells. In contrast, it took 1 kJ/m^2 of UV to cause a similar inhibition of CAT activity in VA cells. F9Vc cells showed a slightly higher CAT activity, if any, than F9 cells (also see Table I). It should be noted that the variation of this assay is 15–20% due to DNA transfection and in vitro CAT analysis.

4. DISCUSSION

In this study, I have reported a cellular protein from F9 parental cells and UV-resistant variant cells that preferentially recognizes UV-damaged DNA. The parameters of both cell lines are summarized in Table I. The F9Vc cells acquired a 6-fold UV resistance compared to the F9 parental cells. The abundance of UVDRP binding activity is about 7-fold higher in F9Vc cells than in F9 cells. The over-production of UVDRP is associated with the UV resistance of F9Vc cells, suggesting that damage recognition may be important for the sensitivity of cells to DNA damage. This is consistent with a rough correlation of the low UVDRP binding activity to UV hypersensitivity in XP cells (this study), and the findings by others that none or a trace of this type of DNA binding occurs in XP group E cells [4,6]. In addition, XP cells exhibited an inefficient reactivation of UV-irradiated plasmid DNA compared to VA cells (see Fig. 2). We have previously demonstrated that UV-resistant HeLa cells acquired a 2-fold increase of repair-associated plasmid reactivation, accompanying a 2-fold increase in UVDRP binding activity [2]. These data suggest a potential correlation between UVDRP and DNA excision repair. Therefore, UVDRP may indirectly contribute to cytotoxicity in some cell lines through DNA repair. However, plasmid reactivation in F9Vc cells is similar to, or only slightly greater, than F9 cells. This raises the possibility that UVDRP binding activity may not be associated with DNA repair in general.

The UVDRP binding activity can be elicited in F9 and F9Vc cells by RA/cAMP. The level of UVDRP increased with cellular differentiation, with a maximum 10- to 15-fold increase after 6 days of induction. The estimated abundance of the maximum UVDRP binding activity in F9 cells is about 0.06 molecules per megabase, which is equivalent to the constitutive UVDRP binding activity of VA cells. It should be noted that 60–70% of F9Vc cells and RA/cAMP-treated F9 cells showed a differentiated parietal endoderm phenotype (data not shown). A prolonged culture of F9 cells probably resulted in a differentiation-like phenotype, as observed in F9Vc cells. Perhaps, the high abundance of UVDRP binding activity in F9Vc cells is acquired from a low abundance in stem cells through a stepwise increase during the process of differentiation. We have recently found that de novo synthesis of a new protein(s) is required for the induction of UVDRP binding activity in mammalian cells by RA/cAMP (C.C.-K.C., unpublished data), suggesting the involvement of a positive regulatory mechanism in the control of the UVDRP induction. It has also been reported by others that a constitutive UVDRP can be induced by pretreatment of monkey cells with UV [16]. The results from our findings suggest that the mammalian UVDRP is regulated by RA/cAMP- or culture-mediated differentiation and by genotoxic stresses. The findings also have the implication that UVDRP may be a potential indication of exogenous and endogenous stresses in cells.

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