Published online May 11, 2004

2652–2659 *Nucleic Acids Research, 2004, Vol. 32, No. 8*
DOI: 10.1093/nar/gkh593

Expression of the adenovirus E4 34k oncoprotein inhibits repair of double strand breaks in the cellular genome of a 293-based inducible cell line

Elham S. Mohammadi, Elizabeth A. Ketner, David C. Johns[1] and Gary Ketner*

Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205, USA and [1]Department of Neurosurgery, The Johns Hopkins Hospital, 422 N Bond Street, Baltimore, MD 21231, USA

Received as resubmission April 15, 2004; Revised and Accepted April 16, 2004

ABSTRACT

The human adenovirus E4 ORF 6 34 kDa oncoprotein (E4 34k), in concert with the 55 kDa product of E1b, prevents concatenation of viral genomes in infected cells, inhibits the repair of double strand breaks (DSBs) in the viral genome, and inhibits V(D)J recombination in a plasmid transfection assay. These activities are consistent with a general inhibition by the E4 34k and E1b 55k proteins of DSB repair by non-homologous end joining (NHEJ) on extrachromosomal substrates. To determine whether inhibition of NHEJ extends to repair of DSBs in the cell chromosome, we have examined the effects of E4 34k on repair of chromosomal DSBs induced by ionizing radiation in a cell line in which E4 34k expression and biological activity is inducible and E1b 55k is produced constitutively. We demonstrate that in this cell line, induction of E4 34k inhibits chromosomal DSB repair. Recently, it has been shown that in infected cells, E4 34k and the adenovirus E1b 55k proteins cooperate to destabilize Mre11 and Rad50, components of mammalian NHEJ systems. Consistent with this, induction of expression of E4 34k in the inducible cell line also reduces the steady state level of Mre11 protein.

INTRODUCTION

The adenoviral E4 34k oncoprotein, the product of E4 open reading frame 6 (ORF 6), mediates a variety of processes in infected cells. For example, through its interaction with the viral E1b 55k protein, E4 34k facilitates accumulation of cytoplasmic viral late mRNA and inhibits the export of cellular mRNA (1–4). The E4 34k/E1b 55K complex induces proteasome-dependent turnover of p53 (5–7), contributing to prevention of premature apoptosis in adenovirus-infected cells (8–10). In cells where E1b 55k is present, E4 34k also inhibits double strand break repair (DSBR) by non-homologous end joining (NHEJ) on a variety of extrachromosomal substrates: it inhibits the formation of concatemers of the viral genome in adenovirus-infected cells, prevents V(D)J recombination of plasmid substrates in a transfection assay, and blocks the repair of double-strand breaks (DSBs) introduced into the viral genome by the HO nuclease (11–14). Interaction with the E1b 55k protein is essential in preventing genome concatenation (14) and V(D)J recombination (Rohleder and Ketner, unpublished). E4 34k also interacts with the host DNA-dependent protein kinase (DNA PK), which is required for DSBR by NHEJ (15,16), and may interfere with NHEJ directly through that interaction (12). Recently, Stracker *et al*. (14) have shown that E4 34k and E1b 55k act to reduce the steady-state levels of the cellular repair proteins Mre11 and Rad50. Homologs of these proteins participate in DSBR both by homologous recombination and NHEJ in yeast (17,18), and reductions in the levels of these proteins provide an alternative or additional mechanism for inhibition of NHEJ in adenovirus-infected cells (14). Inhibition by E4 34k of the NHEJ machinery, either through an interaction with DNA PK or reduction in the levels of the Mre11 or Rad50 proteins, might prevent repair of DSBs in the cellular genome in addition to interfering with DSBR on plasmid and viral DNA substrates. We investigated this possibility by measuring repair of chromosomal breaks created by gamma irradiation of a cell line that inducibly expresses E4 34k.

MATERIALS AND METHODS

Cell culture

293 cells (19) and derivatives were maintained as adherent monolayer cultures in Eagle's Minimal Essential Medium (BioWhittaker), supplemented with 10% fetal bovine serum. All cultures were grown at 37°C in a humidified atmosphere of 5% carbon dioxide, were fed every 2.5 days and subcultured when confluent. The construction of 293VgRXR and 2V6.11, a 293VgRXR derivative that inducibly expresses the E4 34k protein, is described in the Results.

*To whom correspondence should be addressed. Tel: +1 410 955 3776; Fax: +1 410 955 0105; Email: gketner@jhsph.edu
Present address:
Elham S. Mohammadi, Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Comet assay

To detect DNA DSBs on an individual cell basis, 2V6.11 or 293VgRXR cells were cultured in two sets of small straight neck flasks (Nunc) for 24 h. One set (induced) was treated with ponasterone A (1 μg/ml) for 12–16 h and the other (uninduced) was kept as the control. Flasks were gamma-irradiated on ice with a dose of 30 Gy at 10 Gy/min using a Gammacell 1000 ^{137}cesium irradiator (J.L.Shepherd and Associates). Irradiated cells were transferred to a 37°C incubator. At various times after irradiation, neutral comet assays were performed as described (20,21). Briefly, cells were trypsinized, collected by centrifugation and resuspended in 0.5% low melting point (LMP) agarose (Invitrogen) in phosphate-buffered saline (PBS). Approximately 10^5 cells suspended in 50 μl of LMP agarose were applied to microscope slides pre-coated with 1% Seakem ME agarose. After solidifying on a cold block for 5 min, slides were coated with another layer of LMP agarose. Slides were then immersed for 5 min in neutral lysis buffer containing 2% SDS, 25 mM EDTA, 35 mM N-lauroyl sarcosine and 10 mM Tris (pH 8.0). Slides were dipped in PBS briefly and then electrophoresed in a horizontal electrophoresis apparatus at 40 V for 8 min. Slides were stained with SYBR Gold (Molecular Probes) as recommended by the manufacturer and were photographed using a Nikon fluorescent microscope equipped with a CCD camera. Images were analyzed and the level of DNA damage in individual cells was determined by calculation of tail moment (see Results) by means of a public domain macro (22) used with the NIH Image program. NIH Image was developed at the US National Institutes of Health and is available on the internet at http://rsb.info.nih.gov/nih-image/. For the production of Figure 3, representative fields were assembled in Adobe Photoshop and the contrast of some panels was adjusted using the Auto Levels tool.

Statistical analysis of comet assay data

STATA version 6.0 was used to perform a linear regression analysis on the tail moment against group and time for four experimental groups: 293VgRXR uninduced, 293VgRXR induced, 2V6.11 uninduced and 2V6.11 induced. To reduce statistical problems caused by the outliers in the data set (skewed or non-Gaussian distributions) the data were transformed using natural logarithms. The transformed data were fitted to a linear function/model and the logarithm of the tail moment was regressed against group, time and group + time. After correcting for the time point effect on the individual groups, the difference in the natural logarithm of the baseline (293VgRXR uninduced) and every other group was determined.

Growth curves and flow cytometry

Cells were seeded in Nunc 24-well plates or in Nunc 156758 flat-sided tubes at 2.5–4 × 10^5 cells per well or tube. Eighteen hours after seeding, ponasterone A (1 μg/ml) or vehicle (DMSO, final concentration 0.1%) was added in fresh medium to the cultures. If necessary, the medium was replaced at 72 h with medium containing ponasterone A or DMSO as appropriate. At various times after addition of inducer, cells were harvested by trypsinization and counted electronically (Beckman Coulter Z1 cell counter), lysed with PLS (50 mM Tris pH 6.8, 2% SDS, 1% beta-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue), or prepared for flow cytometry by rinsing twice in PBS and resuspending in ice-cold 70% ethanol. After storage for 2–7 days at 4°C in ethanol, cells to be analyzed by flow cytometry were twice rinsed in PBS, treated with 50 μg/ml RNase A at 37°C for 30 min, and suspended in PBS containing 50 μg/ml propidium iodide for 30–90 min. Cytometry was performed on a Becton-Dickenson FACS Calibur.

Immunofluorescence

293VgRXR and 2V6.11 cells were seeded onto glass coverslips 2 days prior to each experiment. When desired, E4 34k expression was induced by treatment with 1 μg/ml ponasterone A 24 h after seeding. Fixation and permeabilization were done essentially as described (23). Cells were fixed with ice-cold methanol for 20 min, and then permeabilized in ice-cold acetone for 10 s. Slides were washed three times in PBS, 5 min each, and were blocked in PBS containing 10% fetal bovine serum (FBS–PBS) for 1 h at room temperature. Cells were then incubated for 1 h at 37°C with polyclonal rabbit serum raised against a C-terminal E4 34k peptide (24), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antiserum (1:200). All antiserum dilutions were in FBS–PBS, and after each blocking and antibody incubation step slides were washed in PBS three times, 5 min each. Slides were mounted in fluorescent mounting medium (Accurate Chemical and Scientific Corp.) and viewed by epifluorescence microscopy.

Immunoblotting

293VgRXR or 2V6.11 cells were seeded at 5 × 10^5 cells per well in 24-well plates (Nunc). After seeding (16–40 h) cultures were treated with 1 μg/ml of ponasterone A or with vehicle (0.1% DMSO). After an additional 24 h incubation, monolayers were harvested either by aspiration of the culture medium and lysis with 200 μl PLS or were infected at an m.o.i. of 3 p.f.u./cell with wild type adenovirus type 5 (Ad5) or with an appropriate E4 deletion mutant (1). Infections were for 3 h in a total volume of 1 ml. Infected cells were harvested 24 h post-infection by aspiration of the culture medium and lysis with 200 μl of PLS. Cell lysates were fractionated on SDS–10% polyacrylamide gels (25) and the proteins were transferred to nitrocellulose (26). The transferred proteins were probed with polyclonal rabbit antiserum against an E4 34k C-terminal peptide, adenovirus late proteins or human Mre11 (Novus Biologicals), and with a monoclonal antibody to actin (BioRad). The secondary antibody was horseradish peroxidase conjugated goat anti-rabbit or donkey anti-mouse IgG (Amersham) and detection was by ECL (Amersham).

RESULTS

Construction of the E4 34k inducible cell line

To simplify investigation of the effect of E4 34k expression on cellular DSBR, we constructed a cell line in which E4 34k is expressed under the control of an ecdysone-inducible promoter. First, HEK 293 cells (19) were transfected with 2.1 μg linearized pVgRXR (Invitrogen) using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

pVgRXR bears a zeocin-resistance selectable marker and constitutively expresses both a modified receptor for the insect hormone ecdysone and the retinoid X receptor. Those proteins confer inducibility by ecdysone or its analogs on promoters bearing cognate hormone-receptor binding sites. Forty-eight hours after transfection, the medium was replaced with medium containing 600 μg/ml zeocin (Invitrogen). Fresh medium was added every 3 days. After 14 days cells were expanded and the mixed population of stable transformants (293VgRXR) was screened for VgRXR expression by transfection with a plasmid containing the enhanced green fluorescent protein (GFP) under the control of an ecdysone inducible promoter [pAdEGI; (27)]. Transfected cells were treated either with 10 μM ponasterone, an ecdysone analog and inducer of the ecdysone promoter, or with vehicle (DMSO) and analyzed by epifluorescence microscopy for the level of GFP. Cells treated with ponasterone were noticeably brighter than the vehicle-treated cells, indicating the presence of a functional ecdysone receptor in most cells. As would be expected, there was considerable variability in the level of induction in this pooled population.

2V6.11 was constructed by transfection of the 293VgRXR population with pEKORF6, a derivative of the plasmid pIndHygro (Invitrogen) that contains Ad5 E4 ORF 6 (which encodes E4 34k) under the control of the ecdysone-inducible promoter present in the plasmid. Hygromycin-resistant clones were selected as recommended by Invitrogen and clones were picked and expanded into cultures. Individual clones were characterized for levels of expression of the E4 34k protein by immunoblotting with an anti-E4 34k antiserum with and without a 24 h induction with 1 μg/ml ponasterone A. Most clones produced E4 34k when induced, and no clone produced detectable E4 34k in the absence of the inducer. The level of E4 34k expression varied substantially from clone to clone, e.g. ~10-fold, as determined by image analysis, for the three clones shown in Figure 1A.

Inducible clones were screened for E4 34k biological activity by testing for the ability to support viral late protein synthesis by H5*dl*1011, H5*dl*1014 or H5*dl*1007, adenovirus E4 deletion mutants defective in viral late gene expression (1). E4 34k-positive clones, with and without prior induction by ponasterone A, were infected at an m.o.i. of 3 p.f.u./cell [titers determined on W162 cells (28)] and extracts were prepared 24 h after infection. The synthesis of viral late protein was assessed by immunoblotting with an antiserum raised against virion proteins (Fig. 1B). Clones expressing the highest induced levels of E4 34k were examined first. Unexpectedly, those clones all significantly complemented the late gene expression defect of E4 deletion mutants, whether or not E4 34k production had been induced (e.g. 2V6.4) (Fig. 1B). As noted above, E4 34k could not be detected by immunoblotting without induction in these lines. We concluded that very low levels of E4 34k are produced in the absence of induction, and that those low levels provided sufficient biological activity to stimulate substantial late gene expression. Therefore, we examined clones that exhibited the lowest induced levels of E4 34k and identified two clones that supported little or no viral late gene expression without induction. One of these, 2V6.11 was chosen for use.

The expression of E4 34k in 2V6.11 was also assessed by immunofluorescence using antiserum directed against E4 34k

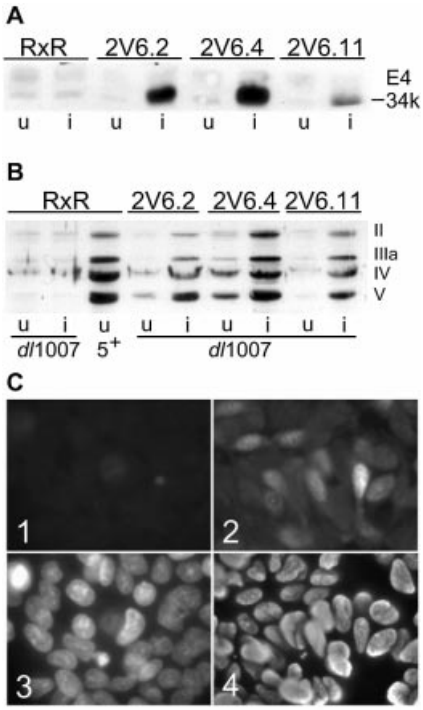

Figure 1. E4 34k expression and biological activity in inducible cell lines. (**A**) Inducible E4 34k expression. Three independent inducible E4 34k cell lines (2V6.2, 2V6.4 and 2V6.11) and the parental population 293VgRxR (RxR) were treated with the inducer ponasterone A or with vehicle (0.1% DMSO). 24 h post-induction, lysates were analyzed by SDS–PAGE and immunoblotting with an anti-E4 34k antibody. u, Protein extracted from uninduced cells; i, protein extracted from induced cells. (**B**) Complementation of late gene expression by inducible cell lines. Inducible and parental cell lines were pretreated for 24 h with ponasterone A (i) or vehicle (u). Cultures then were infected with the E4 deletion mutant H5dl1007 and incubated an additional 24 h in the presence of the inducer or DMSO. The infected cells were lysed and analyzed by SDS–PAGE and immunoblotting with an anti-adenoviral capsid protein antibody. The positions of several viral capsid proteins (II–V) are identified on the right. 5^+, Cells infected with wild-type adenovirus 5. (**C**) Immunofluorescence analysis of E4 34k expression by 2V6.11. 2V6.11 cultures were dually stained with E4 34k antibody and a FITC-conjugated secondary antibody and with the DNA stain Hoechst 33342. Stained cells were examined by fluorescence microscopy and photographed. Top row, E4 34k expression (green fluorescence): 1, uninduced cells; 2, induced cells. Identical exposure settings were used for the two panels. Bottom row, Hoechst fluorescence (blue) of the same fields: 3, uninduced cells; 4, induced cells. The contrast of the lower panels was adjusted using the auto levels tool in Adobe Photoshop.

(Fig. 1C). Modest E4 34k fluorescence was detected in the nucleus of 25–50% of 2V6.11 cells 24 h after induction, and all induced cells showed weak diffuse fluorescence over the entire cell. Uninduced 2V6.11 cells showed no fluorescence either in the nucleus or cytoplasm.

E4 34k, cell growth and the cell cycle

Expression of E4 34k in a 293 cell background has been shown to alter progression through the cell cycle (29). Therefore, we examined cell growth and the cell cycle profile of induced and uninduced 2V6.11 cultures (Fig. 2). Over at least 6 days, cell growth was only slightly retarded by induction of E4 34k expression. Doubling times, derived from exponential curves fitted to the experimental data, were 32 h for uninduced 2V6.11 and 37 h for induced cells. E4 34k was expressed at

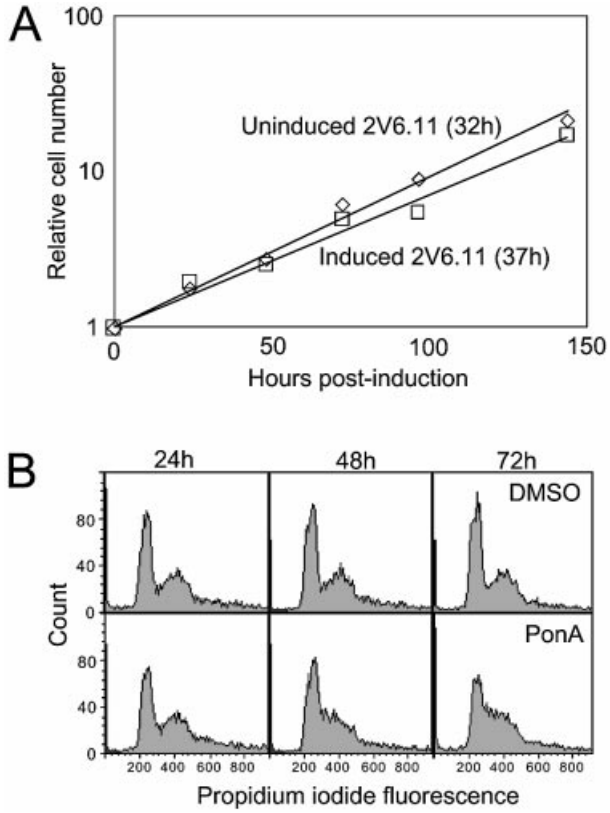

Figure 2. Effect of E4 34k expression on growth and cell cycle progression. (**A**) Growth curves. 2V6.11 cells were grown in the presence either of ponasterone A (PonA) or 0.1% DMSO and the number of cells in induced and uninduced cultures was determined electronically. The curves shown are exponentials fitted to the data by Microsoft Excel. Generation times derived from those curves were 32 h for uninduced cells and 37 h for induced cells. The data for this figure was compiled from three experiments covering different time periods. To permit comparisons of data from experiments with different initial seeding densities, the data are normalized to the cell number present at the time of addition of PonA/DMSO. Diamonds, DMSO-treated cultures; squares, PonA-treated cultures. (**B**) Cell cycle. The DNA content of cells in induced or uninduced cultures was measured by propidium iodide staining and flow cytometry. Times, in hours, after addition of PonA/DMSO are shown above each panel. 20 000 events were counted for each panel.

comparable levels at each time point, as assessed by immunoblotting (data not shown). As has been reported for another inducible line (29), expression of E4 34k substantially increased the proportion of cells in the 2V6.11 population with S-phase DNA contents. Accumulation in S phase was not yet apparent 24 h after induction of E4 34k expression and, since induced cells continue to divide, the S-phase delay must be transient.

Effect of E4 34k on chromosomal DSBR

Since E4 34k prevents DSBR on extrachromosomal substrates and interacts with DNA PK, which is essential for that process, the effect of adenovirus E4 34k on the repair of chromosomal damage induced by ionizing radiation (IR) was investigated. These studies employed the neutral comet assay, a sensitive indicator of DSBs present in single cells (20,21). In the neutral comet assay, individual cells immobilized in agarose are lysed *in situ* under conditions that do not denature chromosomal

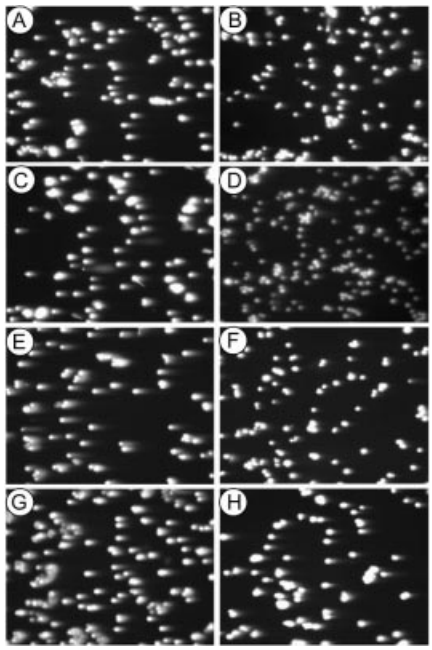

Figure 3. Comet assays of DSBR in irradiated cells. Comet assays were performed on gamma-irradiated 293VgRXR or 2V6.11 cells with or without induction of E4 34k expression by ponasterone A**.** Samples were analyzed immediately after irradiation (left column) or after 10 h repair (right column). (**A**, **B**) 293VgRXR cells, uninduced; (**C**, **D**) 293VgRXR cells, induced; (**E**, **F**) 2V6.11 cells, uninduced; (**G**, **H**) 2V6.11 cells, induced.

DNA and are then subjected briefly to electrophoresis in a low voltage gradient (~2 V/cm). Unbroken chromosomal DNA does not migrate under the electrophoretic conditions employed, but chromosomal DNA containing DSBs induced, for example, by ionizing radiation moves away from its original location at the position of the nucleus of the cell. When stained with a DNA-specific intercalating dye such as SYBR Gold and examined by fluorescence microscopy, cells containing broken DNA resemble comets with a bright head (the original nucleus) and a tail pointing in the direction of electrophoresis (Fig. 3). The relative level of DNA damage in a cell can be quantified by calculation of tail moment, the product of the percentage of DNA in the tail and tail length. Mean tail moment in a population of treated cells is proportional to the dose of DNA-damaging agent and, presumably, to the number of DNA breaks present when the cells are analyzed (30). When repair-competent cells are examined by comet assays, tail moment declines with incubation time after DNA damage is inflicted, reflecting repair of the breaks initially introduced. The half-time for repair (time to reduce tail moment 50%) in normal human fibroblasts is ~3 h as measured by the comet technique (31). Preliminary dose response experiments performed with 293 cells indicated that DNA damage was linear with dose up to at least 100 Gy and that repair could easily be detected by the comet assay at a dose of 25–30 Gy (not shown). A dose of 30 Gy was therefore employed for these studies. Preliminary experiments also showed that induction of E4 34k for 24 h in the absence of irradiation does not elevate tail moment over the level observed in cells that have not been induced for E4 34k expression (see Fig. 4 legend).

Table 1. Regression analysis of comet tail moments in irradiated RXR and 2V6.11 cells

Group	Estimates of the ratio of tail moment measurements by experimental group			
	0 h	2 h	6 h	10 h
RXR induced	1.33 (1.23, 1.44)	0.86 (0.79, 0.93)	1.19 (1.6, 1.8)	1.68 (1.56, 1.81)
2V6.11 uninduced	1.80 (1.68, 1.93)	0.79 (0.73, 0.86)	1.24 (1.15, 1.33)	1.10 (1.03, 1.17)
2V6.11 induced	0.63 (0.58, 0.68)	0.92 (0.85, 0.98)	1.79 (1.69, 1.91)	3.05 (2.83, 2.28)
RXR uninduced	1.00 (NA)	1.00 (NA)	1.00 (NA)	1.00 (NA)

The values given are the ratio of the normalized mean tail moment of the experimental group to that of the control (uninduced 293VgRXR) group. Values in parentheses represent the confidence intervals. R^2 for the model is 73%.

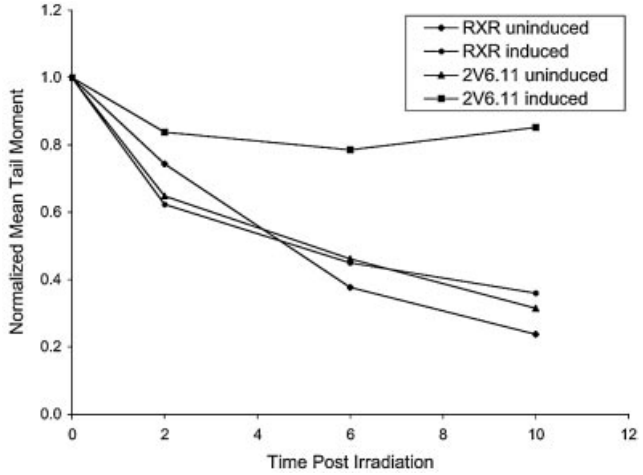

Figure 4. Kinetics of DSBR in 293VgRXR and 2V6.11 cells. Mean tail moment for 2V6.11 and the parental 293VgRXR population, with and without ponasterone A treatment, was determined immediately after irradiation (0) or after 2, 6 and 10 h of incubation at 37°C. Mean tail moment at each time point was normalized against the mean for that group at 0 h post-irradiation. The normalized mean tail moments for unirradiated cells were: 293VgRXR uninduced, 0.111; 293VgRXR induced, 0.167; 2V6.11 uninduced, 0.228; 2V6.11 induced, 0.089.

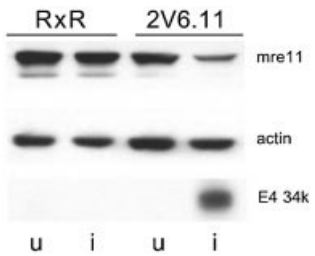

Figure 5. E4 34k expression and steady-state levels of Mre11. Extracts from 2V6.11 or VgRxR cells were analyzed by SDS–PAGE and immunoblotting with anti-Mre11, anti-actin and anti-E4 34k antibodies. u, uninduced; i, induced.

Inspection of comets produced by irradiated cells 10 h after irradiation indicated that cells that did not express E4 34k (parental 293VgRXR and uninduced 2V6.11 cells) repair their DNA efficiently, while the cells expressing the protein (induced 2V6.11 cells) do not (Fig. 3). To quantify repair, representative comet fields were photographed for each cell line 0, 2, 6 and 10 h after irradiation and tail moments were calculated using NIH Image and the Comet plug-in (22). Figure 4 shows the kinetics of repair of DSBs as indicated by mean tail moment over the 10 h repair period for uninduced and induced 293VgRXR and 2V6.11 cells. For the uninduced 2V6.11 cells, mean tail moment, normalized against the mean tail moment at 0 h, declined from 1.0 immediately after irradiation (0 h) to ~0.4 after 10 h of repair. For 2V6.11 cells in which E4 34k expression had been induced with ponasterone A, the mean tail moment for the group changed only from 1.0 at 0 h to ~0.9 at 10 h of repair. Both induced and uninduced control 293VgRXR cells repaired to an extent similar to that of the uninduced 2V6.11 cells. Table 1 shows the results of a comparison of tail moments between uninduced 293VgRXR cells (the control group), induced 293VgRXR cells, and induced and uninduced 2V6.11 cells at various times post-irradiation. At 10 h post-irradiation, the induced 2V6.11 group exhibits a normalized tail moment about three times greater than the control uninduced 293VgRXR group, while no difference is seen between the control and the uninduced 2V6.11 group. The regression model used to compare tail moments among the four experimental groups had an R^2 value of 73%. This suggests that ~73% of the variability in tail moment is predicted by experimental groups and time after irradiation. We conclude that E4 34k strongly interferes with the repair of radiation-induced chromosomal DSBs in a 293 cell background.

Effect of E4 34k on Mre11 levels

Stracker *et al*. (14) have shown that simultaneous expression of E4 34k and E1b 55k reduces the steady-state levels of several proteins involved in DNA repair including Mre11. 2V6.11 cells are derived from 293 cells and constitutively produce E1b 55k. To determine whether induction of E4 34k in 2V6.11 cells destabilizes Mre11, the steady-state level of Mre11 was assessed by immunoblotting of lysates from induced and uninduced 2V6.11 and parental 293VgRXR cells (Fig. 5). The level of Mre11 was substantially reduced in 2V6.11 cells when expression of E4 34k was induced, while the level in 293VgRXR cells was unaffected by the addition of the inducer to the medium. By comparison to an actin loading standard, the Mre11 level observed in uninduced 2V6.11 cells also seemed slightly lower than that in the parental 293VgRXR population, perhaps reflecting a very low level of E4 34k expression in uninduced cells. In our hands, reduction in Mre11 levels in wild-type adenovirus infections is variable and some protein generally remains detectable 24 h post-infection (data not shown).

DISCUSSION

E4 34k, in concert with E1b 55k, inhibits NHEJ on extrachromosomal substrates that include transfected plasmid DNA and the viral genome itself (12–14). Here, we examine repair of chromosomal DSBs in a 293-based cell line in which E4 34k expression and biological activity is inducible. We conclude that the inhibition of DNA repair by E4 34k, presumably in combination with E1b 55k, extends to the cellular genome in addition to extrachromosomal DNA molecules.

The neutral comet assay (20,21) was selected for studying repair of DSBs. The comet assay allows quantification in terms of mean tail moment of the relative levels of chromosomal DNA damage induced in a population of cells by effectors including ionizing radiation (22,30,32–34). DNA repair is reflected as a decrease in mean tail moment with time after DNA damage. In these experiments, DSBs were induced by gamma irradiation in the genome of a cell line (2V6.11) that expresses E4 34k protein inducibly and the E1b 55k protein constitutively. As measured by the comet assay, repair half time was 5–6 h for cells that do not express the adenoviral E4 34k protein (uninduced 2V6.11 cells or the parental cell population), consistent with published figures for wild-type human fibroblasts (~3 h) (31). Induced 2V6.11 cells achieved only ~10% repair over a time course of 10 h, indicating that E4 34k, presumably acting as a member of the E4 34k/E1b 55k complex, inhibits chromosomal DSBR in addition to inhibiting repair of DSBs in extrachromosomal substrates.

DSBs are repaired through two distinct pathways: homologous recombination (HR) and NHEJ. Mammalian cells predominantly use NHEJ to repair DSBs, particularly during G_0 and G_1 (35). In addition, the E4 34k/E1b 55k complex inhibits two other processes dependent upon NHEJ, viral genome concatenation and V(D)J recombination (11,12,14,16,36,37). From those observations and the data presented here, we conclude that induction of the E4 34k expression in 2V6.11 cells prevents chromosomal DSBR primarily as a consequence of inhibition of the NHEJ system. Our data do not rule out the possibility that E 34k or the E4 34k/E1b 55k might also inhibit DSBR by homologous recombination.

NHEJ requires proteins that include DNA PK, DNA ligase IV and the product of the XRCC4 gene (38). E4 34k physically interacts with DNA PK (12), suggesting that E4 34k might directly inhibit an activity of DNA PK required for NHEJ. Apart from the interaction of E4 34k with DNA PK, the E4 34k/E1b 55k complex has been shown to mediate a proteasome-dependent decrease in the levels of Mre11 and Rad50, two members of a physical complex (Mre11-Rad50-Nbs1; MRN) that is involved in DNA damage detection and checkpoint signaling (39,40). The MRN complex may also participate in the NHEJ joining reaction itself. In yeast, the MRN complex is essential for NHEJ (17,41,42) and the *Saccharomyces cerevisiae* MRN complex stimulates joining of linear DNA molecules *in vitro* by yeast DNA ligase IV and the associated protein Lif1 (43). DNA ligase I or IV can also join dissimilar DNA molecules when combined with the human Mre11 protein. That activity is dependent on nuclease activities possessed by Mre11 which appear to form the substrates for the joining reaction (44,45). If Mre11 is required for NHEJ in human cells, the effects of the E4 34k/E1b 55k complex on Mre11 levels also might account for the inhibition of chromosomal DNA repair reported here. Human cells carrying hypomorphic alleles of Mre11 (from ataxia telangectasia-like disorder: ATLD) (46) or Nbs1 (from Nijmegen breakage syndrome: NBS) (47) have been characterized and exhibit chromosome instability, radio-resistant DNA synthesis and sensitivity to ionizing radiation. However, the repair of DNA DSBs is normal in these cells (46,48), indicating that reductions in the activity of the MRN complex are not sufficient to abolish DSBR in human cells. This makes it unlikely that the reduction in Mre11 levels seen in induced 2V6.11 cells is entirely responsible for the inhibition of NHEJ and supports a role in inhibition of chromosomal DSBR for the E4 34k–DNA PK interaction or for inactivation of an unidentified target of the E4 34k/E1b 55k complex.

In yeast, Mre11 nuclease mutants accumulate unprocessed DSBs during meiosis (49,50). The unprocessed DSBs are covalently attached on their 5′ strand to the protein encoded by the Spo11 gene, and the endonuclease activity of Mre11 may be required to remove the protein before exonucleolytic processing can take place (51). The 5′ end of each adenoviral DNA strand is linked to the virally-encoded terminal protein molecule, and Mre11 might act analogously in concatenation by removing terminal protein to produce DNA ends suitable for processing and subsequent joining (14). An Mre11 active-site mutation inhibits viral genome concatenation, consistent with a requirement for nuclease activity in concatemer formation (14). Targeting both DNA PK and the Mre11 complex thus may provide redundant mechanisms to ensure that concatenation is effectively prevented after virus infection. In addition to its potential for inhibiting NHEJ directly, destruction of MRN by the E4 34k/E1b 55k complex prevents activation of the ATM-dependent DNA damage response that otherwise is induced in adenovirus-infected cells (52). This also may increase the success of viral infections by abrogating cell-cycle and apoptotic consequences of the cellular response to DSBs (14,52).

The E4 34k/E1b 55k complex destabilizes p53 in adenovirus-infected cells, antagonizing an E1a-induced activation of p53 that might otherwise induce premature apoptosis (5). p53 destabilization is proteasome-dependent and is mediated by an SCF-type E3 ubiquitin ligase that includes cellular proteins cullin-5, Rbx1/Roc1/Hrt1 and Elongins B and C in addition to the E4 34k/E1b 55k complex (6,7). Apparently, the E4 34k/E1b 55k complex, which binds p53, represents the targeting moiety of the E3 ligase. E4 34k/E1b 55k-dependent modulation of the Mre11-NBS1-Rad50 complex is also accomplished by proteasome-mediated degradation (14), and at least the Mre11 protein is likely to be a target of the E4 34k/E1b 55k E3 ligase (14). Herpes simplex virus 1 targets DNA PK for proteasome-dependent degradation via the immediate early protein vmw110 (53), presumably also to inhibit NHEJ.

We have shown, using an inducible cell line, that inhibition of NHEJ by E4 34k, presumably mediated by the E4 34k/E1b 55k complex, is not restricted to extrachromosomal substrates but extends to the cellular genome as well. Inhibition of chromosomal DNA repair is paralleled by a reduction in steady-state levels of Mre11. These observations are consistent with a model in which DSBR is inhibited by the selective

degradation of components of the DSBR pathway including Mre11, in concert with inhibition of DNA PK through a direct interaction with E4 34k.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service Grant CA-82127 from the National Cancer Institute. The authors wish to thank Lee Martin, Department of Pathology, Johns Hopkins University School of Medicine for valuable assistance with the comet assay.

REFERENCES

1. Bridge,E. and Ketner,G. (1989) Redundant control of adenovirus late gene expression by early region 4. *J. Virol.*, **63**, 631–638.
2. Huang,M.M. and Hearing,P. (1989) Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. *J. Virol.*, **63**, 2605–2615.
3. Pilder,S., Moore,M., Logan,J. and Shenk,T. (1986) The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.*, **6**, 470–476.
4. Sarnow,P., Hearing,P., Anderson,C.W., Halbert,D.N., Shenk,T. and Levine,A.J. (1984) Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J. Virol.*, **49**, 692–700.
5. Querido,E., Marcellus,R.C., Lai,A., Charbonneau,R., Teodoro,J.G., Ketner,G. and Branton,P.E. (1997) Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. *J. Virol.*, **71**, 3788–3798.
6. Harada,J.N., Shevchenko,A., Pallas,D.C. and Berk,A.J. (2002) Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. *J. Virol.*, **76**, 9194–9206.
7. Querido,E., Blanchette,P., Yan,Q., Kamura,T., Morrison,M., Boivin,D., Kaelin,W.G., Conaway,R.C., Conaway,J.W. and Branton,P.E. (2001) Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev.*, **15**, 3104–3117.
8. Dobner,T., Horikoshi,N., Rubenwolf,S. and Shenk,T. (1996) Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. *Science*, **272**, 1470–1473.
9. Martin,M.E. and Berk,A.J. (1998) Adenovirus E1B 55K represses p53 activation in vitro. *J. Virol.*, **72**, 3146–3154.
10. Roth,J., Konig,C., Wienzek,S., Weigel,S., Ristea,S. and Dobbelstein,M. (1998) Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. *J. Virol.*, **72**, 8510–8516.
11. Weiden,M.D. and Ginsberg,H.S. (1994) Deletion of the E4 region of the genome produces adenovirus DNA concatemers. *Proc. Natl Acad. Sci. USA*, **91**, 153–157.
12. Boyer,J., Rohleder,K. and Ketner,G. (1999) Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. *Virology*, **263**, 307–312.
13. Nicolas,A.L., Munz,P.L., Falck-Pedersen,E. and Young,C.S. (2000) Creation and repair of specific DNA double-strand breaks in vivo following infection with adenovirus vectors expressing *Saccharomyces cerevisiae* HO endonuclease. *Virology*, **266**, 211–224.
14. Stracker,T.H., Carson,C.T. and Weitzman,M.D. (2002) Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. *Nature*, **418**, 348–352.
15. Finnie,N.J., Gottlieb,T.M., Blunt,T., Jeggo,P.A. and Jackson,S.P. (1996) DNA-dependent protein kinase defects are linked to deficiencies in DNA repair and V(D)J recombination. *Phil. Trans. R. Soc. Lond. B Biol. Sci.*, **351**, 173–179.
16. Jackson,S.P. and Jeggo,P.A. (1995) DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. *Trends Biochem. Sci.*, **20**, 412–415.
17. Moore,J.K. and Haber,J.E. (1996) Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 2164–2173.
18. Boulton,S.J. and Jackson,S.P. (1998) Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. *EMBO J.*, **17**, 1819–1828.
19. Graham,F.L., Smiley,J., Russell,W.C. and Nairn,R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.*, **36**, 59–74.
20. Singh,N.P., McCoy,M.T., Tice,R.R. and Schneider,E.L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.
21. Ostling,O. and Johanson,K.J. (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.*, **123**, 291–298.
22. Helma,C. and Uhl,M. (2000) A public domain image-analysis program for the single-cell gel-electrophoresis (comet) assay. *Mutat. Res.*, **466**, 9–15.
23. Maser,R.S., Monsen,K.J., Nelms,B.E. and Petrini,J.H. (1997) hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks. *Mol. Cell. Biol.*, **17**, 6087–6096.
24. Boivin,D., Morrison,M.R., Marcellus,R.C., Querido,E. and Branton,P.E. (1999) Analysis of synthesis, stability, phosphorylation and interacting polypeptides of the 34-kilodalton product of open reading frame 6 of the early region 4 protein of human adenovirus type 5. *J. Virol.*, **73**, 1245–1253.
25. Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
26. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Johns,D.C., Marx,R., Mains,R.E., O'Rourke,B. and Marban,E. (1999) Inducible genetic suppression of neuronal excitability. *J. Neurosci.*, **19**, 1691–1697.
28. Weinberg,D.H. and Ketner,G. (1983) A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2. *Proc. Natl Acad. Sci. USA*, **80**, 5383–5386.
29. Grifman,M., Chen,N.N., Gao,G.P., Cathomen,T., Wilson,J.M. and Weitzman,M.D. (1999) Overexpression of cyclin A inhibits augmentation of recombinant adeno-associated virus transduction by the adenovirus E4orf6 protein. *J. Virol.*, **73**, 10010–10019.
30. Olive,P., Banath,J. and Durand,R. (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the 'comet' assay. *Radiat. Res.*, **122**, 86–94.
31. Wang,H., Guan,J., Perrault,A.R., Wang,Y. and Iliakis,G. (2001) Replication protein A2 phosphorylation after DNA damage by the coordinated action of ataxia telangiectasia-mutated and DNA-dependent protein kinase. *Cancer Res.*, **61**, 8554–8563.
32. Ashby,J., Tinwell,H., Lefevre,P.A. and Browne,M.A. (1995) The single cell gel electrophoresis assay for induced DNA damage (comet assay): measurement of tail length and moment. *Mutagenesis*, **10**, 85–90.
33. McMillan,T.J., Tobi,S., Mateos,S. and Lemon,C. (2001) The use of DNA double-strand break quantification in radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.*, **49**, 373–377.
34. Olive,P.L., Durand,R.E., Jackson,S.M., Riche,J.C.L., Luo,C., Ma,R., McLaren,D.B., Aquino-Parsons,C., Thomson,T.A. and Trotter,T. (1999) The comet assay in clinical practice. *Acta Oncol.*, **38**, 839–844.
35. Jackson,S.P. (2002) Sensing and repairing DNA double-strand breaks. *Carcinogenesis*, **23**, 687–696.
36. Gellert,M. (2002) V(D)J recombination: RAG proteins, repair factors and regulation. *Annu. Rev. Biochem.*, **71**, 101–132.
37. Jeggo,P.A., Taccioli,G.E. and Jackson,S.P. (1995) Menage a trois: double strand break repair, V(D)J recombination and DNA-PK. *Bioessays*, **17**, 949–957.
38. Jeggo,P.A. (1998) Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. *Radiat. Res.*, **150**, S80–S91.
39. Carney,J.P., Maser,R.S., Olivares,H., Davis,E.M., Le Beau,M., Yates,J.R.,3rd, Hays,L., Morgan,W.F. and Petrini,J.H. (1998) The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell*, **93**, 477–486.
40. D'Amours,D. and Jackson,S.P. (2002) The Mre11 complex: at the crossroads of DNA repair and checkpoint signalling. *Nat. Rev. Mol. Cell. Biol.*, **3**, 317–327.
41. Kramer,K.M., Brock,J.A., Bloom,K., Moore,J.K. and Haber,J.E. (1994) Two different types of double-strand breaks in *Saccharomyces cerevisiae*

are repaired by similar RAD52-independent, nonhomologous recombination events. *Mol. Cell. Biol.*, **14**, 1293–1301.
42. Usui,T., Ohta,T., Oshiumi,H., Tomizawa,J., Ogawa,H. and Ogawa,T. (1998) Complex formation and functional versatility of Mre11 of budding yeast in recombination. *Cell*, **95**, 705–716.
43. Chen,L., Trujillo,K., Ramos,W., Sung,P. and Tomkinson,A.E. (2001) Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. *Mol. Cell*, **8**, 1105–1115.
44. Paull,T.T. and Gellert,M. (2000) A mechanistic basis for Mre11-directed DNA joining at microhomologies. *Proc. Natl Acad. Sci. USA*, **97**, 6409–6414.
45. Paull,T.T. and Gellert,M. (1998) The 3′ to 5′ exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol. Cell*, **1**, 969–979.
46. Stewart,G.S., Maser,R.S., Stankovic,T., Bressan,D.A., Kaplan,M.I., Jaspers,N.G., Raams,A., Byrd,P.J., Petrini,J.H. and Taylor,A.M. (1999) The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell*, **99**, 577–587.
47. Varon,R., Vissinga,C., Platzer,M., Cerosaletti,K.M., Chrzanowska,K.H., Saar,K., Beckmann,G., Seemanova,E., Cooper,P.R., Nowak,N.J. *et al.* (1998) Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell*, **93**, 467–476.
48. Kraakman-van der Zwet,M., Overkamp,W.J., Friedl,A.A., Klein,B., Verhaegh,G.W., Jaspers,N.G., Midro,A.T., Eckardt-Schupp,F., Lohman,P.H. and Zdzienicka,M.Z. (1999) Immortalization and characterization of Nijmegen Breakage syndrome fibroblasts. *Mutat. Res.*, **434**, 17–27.
49. Furuse,M., Nagase,Y., Tsubouchi,H., Murakami-Murofushi,K., Shibata,T. and Ohta,K. (1998) Distinct roles of two separable *in vitro* activities of yeast Mre11 in mitotic and meiotic recombination. *EMBO J.*, **17**, 6412–6425.
50. Tsubouchi,H. and Ogawa,H. (1998) A novel mre11 mutation impairs processing of double-strand breaks of DNA during both mitosis and meiosis. *Mol. Cell. Biol.*, **18**, 260–268.
51. Moreau,S., Ferguson,J.R. and Symington,L.S. (1999) The nuclease activity of Mre11 is required for meiosis but not for mating type switching, end joining, or telomere maintenance. *Mol. Cell. Biol.*, **19**, 556–566.
52. Carson,C.T., Schwartz,R.A., Stracker,T.H., Lilley,C.E., Lee,D.V. and Weitzman,M.D. (2003) The Mre11 complex is required for ATM activation and the G2/M checkpoint. *EMBO J.*, **22**, 6610–6620.
53. Parkinson,J., Lees-Miller,S.P. and Everett,R.D. (1999) Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. *J. Virol.*, **73**, 650–657.