cules is kept constant.

Because the structural and functional properties of telomeres appear to be highly conserved, our findings may be relevant to telomere length regulation in humans, which has been associated with aging and cancer (*29*). The discovery of human proteins that bind specifically to telomeric repeats (*30*), and more recent functional studies (*31*) on one of these proteins, TRF1, suggest that a protein-counting mechanism similar to that described here may regulate telomere length in human cells.

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14. Plasmids pSB362 (Gbd alone), pSB136 (Gbd/Rap1), and pSB341 (Gbd/rap1-12) are based on pRS313 (*HIS3 CENVI*) and contain sequences encoding Gbd fused to the *RAP1* promoter and the 3′ end of *RAP1* from codon 653 (*21*). Plasmids sp138 (Gbd-Sir4) and sp131 (Gbd-Sir3) are described in (*24*), and plasmid pKL5 (Gbd-Sir1) in (*23*). Plasmids sp17 (*RAP1*), sp103 (*rap1-12*), and sp19 (*rap1-825**) are based on pRS313, with the *HIS3* gene disrupted as follows: plasmids LSD135, pSB328 (*21*), and PM585 (*19*) were cut with Nde I, end-filled with Klenow, and religated to give plasmids sp17, sp103, and sp19, respectively. Plasmids sp179 (one UAS_G site) and sp180 (two UAS_G sites) were constructed as follows: UAS_G oligonucleotides (*21*) were end-filled, ligated with a Bgl II linker, cut with Bgl II, and inserted into sp59 cut with Bam HI. A third UAS_G site was ligated with Bgl II–cut sp180 to give plasmid sp181. Lev130, Lev132, and Lev134 result from the transformation of Lev95 with the linearized plasmids sp179, sp180, and sp181, respectively.
15. Overexpression of the COOH-terminal half of Rap1p from a high–copy number plasmid was reported to cause telomere lengthening (*5*) [C. F. J. Hardy, thesis, Columbia University (1991)]. In our experiments, the Gbd/Rap1 hybrid was expressed on a centromere plasmid by means of the *RAP1* promoter, which most likely explains its lack of effect on telomere length in the absence of UAS_G sites.
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26. In *rap1*^{t} cells, which lack most of the COOH-terminal region of Rap1p, targeting Rap1p COOH-terminal domains to a telomere containing four UAS_G sites is still insufficient to reduce telomere length (*17*); this indicates that a threshold number of Rap1p COOH-terminal domains, not obtained under these conditions, is required to effectively inhibit telomere elongation.
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Defective Transcription-Coupled Repair of Oxidative Base Damage in Cockayne Syndrome Patients from XP Group G

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In normal human cells, damage due to ultraviolet light is preferentially removed from active genes by nucleotide excision repair (NER) in a transcription-coupled repair (TCR) process that requires the gene products defective in Cockayne syndrome (CS). Oxidative damage, including thymine glycols, is shown to be removed by TCR in cells from normal individuals and from xeroderma pigmentosum (XP)-A, XP-F, and XP-G patients who have NER defects but not from XP-G patients who have severe CS. Thus, TCR of oxidative damage requires an XPG function distinct from its NER endonuclease activity. These results raise the possibility that defective TCR of oxidative damage contributes to the developmental defects associated with CS.

Nucleotide excision repair is an evolutionarily conserved pathway by which cells remove a wide variety of helix-distorting lesions from DNA. It is a complex multiprotein process involving dual incisions on either side of the lesion and removal of an oligonucleotide containing the damage (*1*). Defects in any of seven different human genes (*XPA* through *XPG*) encoding proteins with functions in the early steps of NER result in the hereditary disease xeroderma pigmentosum, characterized by sensitivity to sunlight, marked skin changes in exposed areas, and extreme susceptibility to skin cancer. Cells from patients with another hereditary disease, Cockayne syndrome, are specifically defective in the preferential removal of lesions from transcribed strands of active genes by a TCR process that has

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been regarded as a subclass of NER (*2*). Aside from sun sensitivity, however, there is little overlap in the clinical symptoms of XP and CS, with the latter including characteristic dwarfing with skeletal abnormalities and obligate neurological dysfunction (*3*). Two genes required for TCR, *CSA* and *CSB*, have been cloned, but their precise roles remain to be elucidated (*4*).

XP and CS are usually genetically as well as clinically distinct, with the great majority of CS patients belonging to the CS-A or CS-B complementation groups. However, a few CS patients belong to XP complementation groups B, D, or G (*5*). Both XPB and XPD are helicases and are subunits of the transcription factor TFIIH, which is required both for initiation of transcription by RNA polymerase II and for NER, in which it presumably opens the DNA helix near the lesion (*1*). The *XPG* gene (*6*) encodes a large protein with an endonuclease activity required for making the 3′ incision in NER (*1, 7*). The dual role of XPB and XPD in transcription and in NER provides an explanation for the essential function of their homologs in *Saccharomyces cerevisiae* and has led to the suggestion that CS may be a transcription disorder rather than a repair-deficiency disease (*5*). The absence of CS in XP-A patients completely lacking in NER due to defective damage recognition is consistent with this idea. However, this model does not readily apply to XPG, because it has no known function in transcription and because disruptions of *RAD2*, encoding its *S. cerevisiae* homolog, are viable.

We previously showed that both normal human cells and cells with mutations in the *XPA* gene preferentially remove ionizing radiation–induced damage from transcribed strands but that *CSA* and *CSB* mutant cells are defective in this TCR process (*8*). This observation suggested that lesion removal by a pathway other than NER might also be coupled to transcription and that such a repair pathway might be defective in CS. To examine the possible relation between TCR of ionizing radiation damage, CS, and NER functions, we measured TCR in x-irradiated XP-G mutant cell strains from patients with and without clinical symptoms of CS.

There is considerable clinical heterogeneity among the few known XP-G patients. XP125LO is one of two siblings with a mild XP phenotype but no other clinical symptoms (*9*). Causative mutations have been identified in both *XPG* alleles, with the maternal allele resulting in a full-length protein with a missense mutation in the presumed active site of the endonuclease (*10, 11*). In contrast, three patients with severe infantile CS—XPCS1LV, XPCS2LV, and 94RD27 (*12*)—have mutations that would lead to truncated XPG proteins (*11*). Strand-specific repair in the active metallothionein IA (*MT1A*) gene was measured in x-irradiated primary fibroblasts from all these XP-G patients and from a normal individual (Fig. 1). In normal fibroblasts there was a much higher rate of repair in the transcribed strand (TS) than in the nontranscribed strand (NTS) or in the genome overall. In contrast, all three XP-G/CS cell strains (XPCS1LV, XPCS2LV, and 94RD27) were completely defective in this preferential repair, with transcribed strands being repaired at the same rate as NTS or total DNA. Moreover, repair in total DNA was reduced relative to that in normal cells (compare right and left panels in Fig. 1B).

As in XP-A cells (*8*), TCR was normal in x-irradiated XP-F cells (Fig. 1, left), which are defective in the endonuclease activity that incises on the 5′ side of NER-recognized lesions (*13*). Repair of x-ray–induced damage in the XP-G mutant XP125LO was also identical to that in normal cells (Fig. 1B, middle). Although full-length XPG is produced in XP125LO cells (*11*), their *XPG* mutations completely prevent NER by several criteria. The cells have low levels of unscheduled DNA synthesis after ultraviolet (UV) irradiation (*6*), and XP125LO extracts neither support repair synthesis on UV-irradiated DNA nor incise cisplatin-adducted DNA (*13, 14*). Furthermore, there is no detectable removal of cyclobutane pyrimidine dimers from either strand of the *MT1A* gene in UV-irradiated XP125LO cells (*11*). Thus, defects in three different genes that result in inactivation of NER (*XPA*, *XPF*, conservative mutation in *XPG*) do not affect TCR of ionizing radiation damage, whereas mutations that severely truncate the XPG protein eliminate it.

Oxidatively damaged bases are the most abundant class of damage from ionizing radiation or other processes that generate reactive oxygen species, including normal cellular metabolism (*15*). One of these is thymine glycol (Tg), a prevalent, stable, and lethal lesion that blocks transcription (*16*). To determine whether the ionizing radiation repair defect in the XP-G/CS strains affected removal of Tg, we quantified its production and removal with a monoclonal antibody (*17*). In x-irradiated normal and XP-F mutant cells, Tg was removed rapidly [half-time ($t_{1/2}$) < 45 min] from the genome overall (Fig. 2, left). Remov-

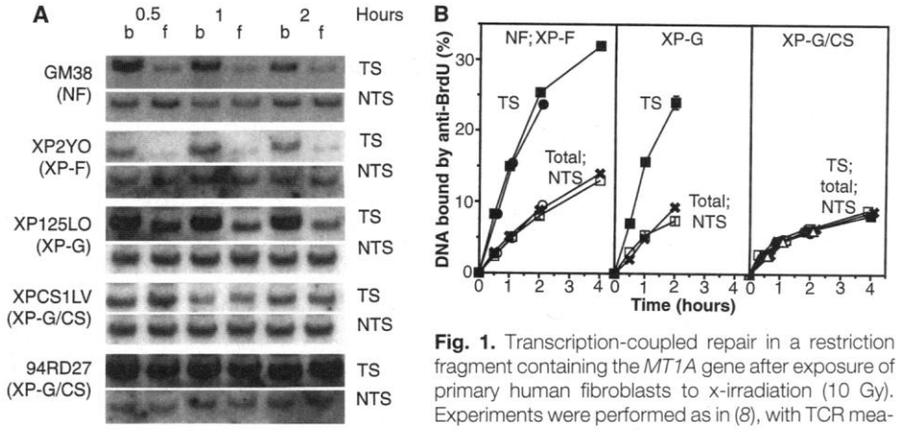

Fig. 1. Transcription-coupled repair in a restriction fragment containing the *MT1A* gene after exposure of primary human fibroblasts to x-irradiation (10 Gy). Experiments were performed as in (*8*), with TCR measured by an immunological assay detecting incorporation of BrdU in repair patches. Briefly, cultured fibroblasts labeled with [^{3}H]thymidine were irradiated and allowed to repair in the presence of BrdU. Purified DNA was digested with Eco RI and incubated with a monoclonal antibody against BrdU (anti-BrdU). Total repair was assessed by the amount of ^{3}H-labeled DNA bound. Gene-specific repair was determined by subjecting to electrophoresis on agarose gels equal amounts of DNA from the bound and free fractions and then quantitating the intensity of hybridization to specific restriction fragments of an RNA probe for either strand of the human metallothionein (*MT*) gene family. Results are presented for the *MT1A* gene, which is located on a 10-kb restriction fragment and which is transcribed at basal levels in all cell lines and tissues where it has been examined. The value for the intensity of hybridization to the fragment of interest is multiplied by the proportion of DNA in the bound or free fractions to determine the total amount of the gene in each fraction. The percentage of the gene containing BrdU is then calculated from the total amount of the gene in the bound fraction divided by the total amount of the gene in the bound plus free fractions. The autoradiograms in (**A**) are from representative experiments; values plotted in (**B**) are means of one to five such independent experiments for each cell strain as indicated, with overlapping symbols displaced for clarity. Error bars (SEM) in most cases are smaller than the symbol. b, DNA bound by anti-BrdU; f, DNA free of antibody; TS, transcribed strand; NTS, nontranscribed strand. (B) Filled symbols, TS; open symbols, NTS; X, total DNA. (Left) NF (normal fibroblasts, GM38 from the NIGMS Human Mutant Cell Repository, Camden, NJ), squares (five experiments); XP-F strain XP2YO (GM4313), circles (one experiment). (Middle) XP-G strain XP125LO, squares (two experiments). (Right) Three XP-G/CS cell strains: XPCS1LV, squares (three experiments); XPCS2LV, circles (one experiment); 94RD27, triangles (one experiment).

al was normal in the XP-G strain XP125LO but was significantly slower in the XP-G/CS strain XPCS1LV. Damage induced by treatment of cells with H_2O_2 to produce an ~100-fold higher frequency of Tg was also removed efficiently both by normal cells and by XP-A, XP-F, and CS-B mutant cells (Fig. 2, right). In all three H_2O_2-treated XP-G/CS mutants Tg was removed at about half the initial rate of the normal cells, whereas in the XP-G mutant XP125LO the rate of Tg removal was normal. These results suggest that the reduced level of total repair in the x-irradiated XP-G/CS mutants (Fig. 1) reflects impaired removal of Tg and related damage. They further suggest the existence of two mechanisms for Tg removal in the genome overall. One of these requires a function of XPG other than incision, which is defective in both the XP-G and the XP-G/CS mutants (*11*), but neither requires NER.

To examine gene-specific removal of Tg, we purified DNA from H_2O_2-treated cells, incubated it with antibodies to Tg (anti-Tg), and separated the antibody-bound DNA from free DNA not containing Tg (*18*). Analysis of repair in the active *MTIA* gene of normal cells by hybridization of strand-specific probes revealed that Tg was removed more rapidly from the transcribed strand than from the nontranscribed strand or from the total DNA (Fig. 3). This was also true for both XP-A and XP-F mutants as well as for the XP-G strain XP125LO, all of which are defective in NER. In contrast, there was no preferential removal of Tg in any of the XP-G/CS mutants or in the CS-B mutant CS1AN, which is also defective in TCR of ionizing-radiation damage (*8*). Thus, preferential removal of Tg from transcribed strands of active genes correlates with TCR of ionizing radiation damage, and neither proceeds by NER.

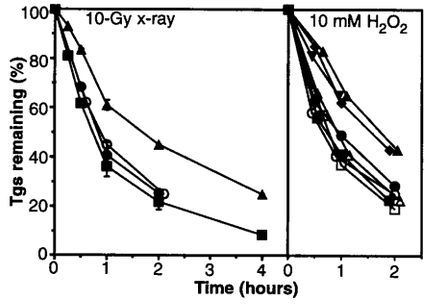

Fig. 2. Global (genome overall) removal of Tg after exposure of human fibroblasts to x-rays (10 Gy) or hydrogen peroxide (10 mM). Cells were treated with H_2O_2 in culture medium for 15 min at 37°C. After removal of the medium, cells were washed with buffered saline and either harvested immediately by lysis or incubated in medium to allow repair. The Tg content of purified DNA was analyzed by an enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody to Tg (anti-Tg) (*17*). X-rays (10 Gy) induced an average of 0.95 Tg per 10^6 bases (SD = 0.12; 13 independent determinations), whereas the H_2O_2 treatment induced an average of 1.1 Tg per 10 kb (SD = 0.29; 16 independent determinations). Values for Tg removal are means of determinations from one to five separate experiments per strain; replicate determinations from the same experiment for any given sample were typically within 5% of each other. Overlapping symbols have been displaced for clarity. NF (normal fibroblasts, GM38), ■; XP-G (XP125LO), ●; XP-G/CS (XPCS1LV), ▲; XP-G/CS (XPCS2LV), ◆; XP-G/CS (94RD27), ▼; XP-A (XP12BE, GM5509), □; XP-F (XP2YO, GM4313), ○; CS-B (CS1AN, GM739), △.

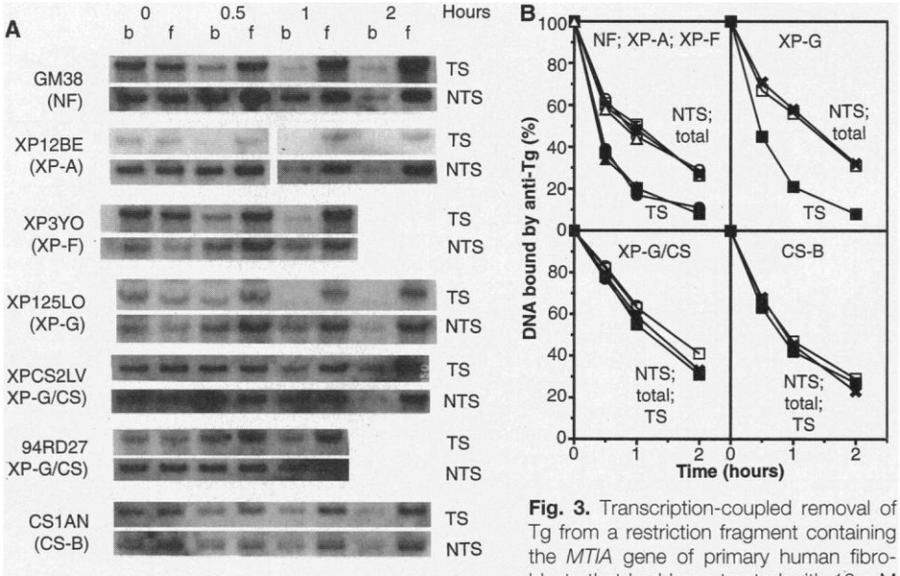

Fig. 3. Transcription-coupled removal of Tg from a restriction fragment containing the *MTIA* gene of primary human fibroblasts that had been treated with 10 mM H_2O_2. Purified DNA was digested with Eco RI, incubated with anti-Tg, and the extent of repair determined (*18*). (**A**) Representative autoradiograms. b, DNA bound by the antibody; f, DNA free of antibody; TS, transcribed strand; NTS, nontranscribed strand. (**B**) Plots of values determined from one to three such experiments for each strain; in most cases when only one experiment was performed, duplicate samples were analyzed. Filled symbols, TS; open symbols, NTS; X, total DNA. (Upper left) NF (normal fibroblasts, GM38), squares; XP-A (XP12BE, GM5509), circles; XP-F (XP3YO, GM3542; this patient is the grand-nephew of XP2YO used in Figs. 1 and 3), triangles. (Upper right) XP-G (XP125LO), squares. (Lower left) XP-G/CS strains (XPCS2LV), squares; (94RD27), circles. (Lower right) CS-B (CS1AN, GM739), squares.

Although repair of a subclass of lesions induced by ionizing radiation depends on XP factors (*19*), most ionizing radiation–induced base lesions and single-strand breaks are thought to be repaired by the process of base excision repair (BER), in which damaged bases are removed by specific *N*-glycosylases, followed by strand scission at the resulting apurinic/apyrmidinic (AP) site by an AP endonuclease (*20*). Presumably, Tg removal occurs by glycosylase-AP endonuclease action that is facilitated by XPG. The hypersensitivity to ionizing radiation, H_2O_2, and oxidative stress of human cells deficient in AP endonuclease is consistent with this hypothesis (*21*). The roles of XPG and of CSB in removal of oxidative base damage are clearly different, because XPG participates in global removal as well as being essential for TCR, whereas absence of CSB affects only the TCR process (Figs. 2 and 3). The XPG homolog RAD2 similarly participates in TCR of Tg in *S. cerevisiae*, although its requirement is not as absolute as for XPG in human cells, and the mechanism in yeast is also distinct from NER (*22*). Within XP group G, defects in TCR correlate with severe truncations of the XPG protein (*11*), consistent with the idea that certain domains of XPG are essential for protein-protein interactions that are required for efficient and preferential removal of Tg. Recent studies of the mechanism of NER have shown that the 5′-incisions in NER by ERCC1/XPF do not occur in the absence of XPG and have implicated XPG in assembly of a preincision complex in NER (*23*). Our data suggest that XPG may have a similar role in BER as well as interacting with proteins required for TCR, such as TFIIH, CSA, or CSB (*24*).

In the case of genetic diseases for which the altered gene product has been identified, it is frequently not apparent how the clinical manifestations relate to the known biochemical defect. This is particularly so for CS, for which the difficulty is compounded by the fact that mutations in five different genes can give rise to the clinical syndrome. Among XP-G patients, we find a

direct correlation between the clinical presence of CS and mutations that inactivate a second function of XPG, its role in TCR of oxidative damage. Mutations in CSA and CSB also affect TCR of oxidative damage, further suggesting that this repair defect may relate directly to the clinical outcome. The unusual severity of the syndrome in the XP-G/CS patients, all of whom died in infancy or early childhood, may reflect the additional deficit in global removal of oxidative damage from the genome. The simplest interpretation of our results is that failure to rapidly repair endogenous oxidative base damage in critical active genes, rather than subtle transcription defects, results in the developmental defects of CS.

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Regulation of Replicon Size in *Xenopus* Egg Extracts

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Once a specific number of cells have been produced in the early *Xenopus laevis* embryo, replicon size during the S phase of the cell cycle increases. Here, it is reported that similar increase in replicon size occurred when the concentration of nuclei in replication-competent *Xenopus* egg extracts exceeded a critical threshold. In this system, the origin recognition complex (ORC) did not become stoichiometrically limiting for initiation, and similar amounts of this complex bound to chromatin regardless of replicon size. These data suggest that in early development, an unidentified factor controls how many preformed ORC-DNA complexes initiate DNA replication.

In organisms such as *Xenopus* and *Drosophila*, the S phase (during which DNA is replicated) lasts only a few minutes in embryonic cells whereas it takes several hours in somatic cells. This difference exists because embryonic replicons are much smaller than somatic replicons (*1*, *2*). The transition from small to large replicons probably takes place at about the 12th cell division cycle after fertilization (*3*–*5*), at the time of the mid-blastula transition (MBT). The mechanism regulating replicon size is not known. One clue is that the MBT occurs after a critical number of nuclei accumulate in the embryo (*3*, *4*), suggesting that the nucleo-cytoplasmic ratio might regulate replicon size, perhaps through depletion of an initiation factor (*1*, *3*, *6*). To study the mechanism controlling replicon size, we used *Xenopus* egg extracts, which assemble *Xenopus* sperm chromatin into nuclei that undergo one complete round of DNA replication (*7*). We attempted to modulate replicon size in vitro by altering the nucleocytoplasmic ratio in these egg extracts.

The time required for nuclei to enter mitosis in *Xenopus* egg extracts increases with increasing concentrations of nuclei, perhaps reflecting an increase in the length of the S phase (*8*). To directly measure the effect of the nuclei concentration on the length of the S phase, we formed nuclei at 2000 and 10,000 per microliter and synchronized them at the start of S phase with cytosine β-D-arabinofuranoside 5′-triphosphate (Ara-C), a deoxycytidine triphosphate (dCTP) analog that blocks elongation through its action on DNA polymerase but does not prevent initiation (*9*) (and see below). After incubating the nuclei for 40 min with Ara-C, we added excess dCTP to reverse the inhibition by Ara-C and measured the time subsequently required for completion of DNA replication. The length of the S phase in reactions with 10,000 nuclei/μl was eight times that in reactions with 2000 nuclei/μl (Fig. 1A). However, the addition of dCTP did not completely reverse the effect of the Ara-C as the rate of polymerization was about 44% the rate in the absence of Ara-C (decreased by a factor of ~2.25) (*10*). Correcting for this, the actual length of the S phase was ~10 min at 2000 nuclei/μl and ~80 min at 10,000 nuclei/μl.

We next measured the length of the S phase over a range of nuclei concentrations and found it was constant at ~10 min between 125 and 2000 nuclei/μl and increased significantly at concentrations greater than 2000 nuclei/μl, reaching 80 min at 10,000 nuclei/μl (Fig. 1B). Similarly, the S phase in developing *Xenopus* embryos is 10 to 15 min during the divisions preceding the MBT, after which it gradually increases (*3*, *5*). In the absence of Ara-C, the S phase also lengthens at concentrations of nuclei >2000 per micro-

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