

# Involvement of Rel/Nuclear Factor- $\kappa$ B Transcription Factors in Keratinocyte Senescence

David Bernard,<sup>1</sup> Karo Gosselin,<sup>1</sup> Didier Monte,<sup>1</sup> Chantal Vercamer,<sup>1</sup> Fatima Bouali,<sup>1</sup> Albin Pourtier,<sup>2</sup> Bernard Vandebunder,<sup>1</sup> and Corinne Abbadie<sup>1</sup>

<sup>1</sup>UMR 8117 CNRS-Institut Pasteur de Lille-Université Lille 1, Institut de Biologie de Lille, Lille Cedex, France, and <sup>2</sup>Laboratoire de Biologie du Développement, Université Lille 1, Villeneuve d'Ascq Cedex, France

## ABSTRACT

After a finite doubling number, normal cells become senescent, *i.e.*, nonproliferating and apoptosis resistant. Because Rel/nuclear factor (NF)- $\kappa$ B transcription factors regulate both proliferation and apoptosis, we have investigated their involvement in senescence. cRel overexpression in young normal keratinocytes results in premature senescence, as defined by proliferation blockage, apoptosis resistance, enlargement, and appearance of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) activity. Normal senescent keratinocytes display a greater endogenous Rel/NF- $\kappa$ B DNA binding activity than young cells; inhibiting this activity in presenescent cells decreases the number of cells expressing the SA- $\beta$ -Gal marker. Normal senescent keratinocytes and cRel-induced premature senescent keratinocytes overexpressed manganese superoxide dismutase (MnSOD), a redox enzyme encoded by a Rel/NF- $\kappa$ B target gene. MnSOD transforms the toxic O<sub>2</sub><sup>-</sup> into H<sub>2</sub>O<sub>2</sub>, whereas catalase and glutathione peroxidase convert H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O. Neither catalase nor glutathione peroxidase is up-regulated during cRel-induced premature senescence or during normal senescence, suggesting that H<sub>2</sub>O<sub>2</sub> accumulates. Quenching H<sub>2</sub>O<sub>2</sub> by catalase delays the occurrence of both normal and premature cRel-induced senescence. Conversely, adding a nontoxic dose of H<sub>2</sub>O<sub>2</sub> to the culture medium of young normal keratinocytes induces a premature senescence-like state. All these results indicate that Rel/NF- $\kappa$ B factors could take part in the occurrence of senescence by generating an oxidative stress via the induction of MnSOD.

## INTRODUCTION

When explanted from tissue into *in vitro* culture, normal cells undergo a finite number of divisions and thereafter enter a nonreplicative state termed replicative or cellular senescence. This phenomenon was first described for human fibroblasts (1) and extended to a variety of cell types and species, from yeast to mammals (2, 3). Human senescent cells display a characteristic enlarged and spread morphology, accompanied by an accumulation of lipofuscin and a significant percentage of polynucleation (4–11). They are irreversibly cell cycle arrested, preferentially but not exclusively at the G<sub>1</sub>-S boundary (12), and they are apoptosis resistant (13–16). They express a particular senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) activity at pH 6 that represents the most universal molecular biomarker of senescence (17). Two main mechanisms were shown to promote senescence: telomere erosion and cumulative oxidative damage (18). The most widely accepted model assumes that telomeres shorten

because of the so-called end-replication problem; this shortening engages a DNA damage signal that leads to proliferation blockage (19). Reactive oxygen species (ROS) produced along all cell life constantly attack DNA and other macromolecules, resulting in accumulation of undegradable oxidized material (lipofuscin) and DNA damage-induced cell cycle arrest (11, 20–22).

Rel/nuclear factor (NF)- $\kappa$ B proteins are ubiquitous transcription factors recognized as central regulators of cell growth. In vertebrates, the Rel/NF- $\kappa$ B family comprises five members able to form homo- or heterodimers: cRel; RelA (p65); RelB; NF- $\kappa$ B1 (p50); and NF- $\kappa$ B2 (p52). Rel/NF- $\kappa$ B dimers are constitutively present in the cytoplasm of numerous cell types, sequestered by I $\kappa$ B proteins. On stimulation, I $\kappa$ B proteins are phosphorylated, ubiquitinated, and degraded, therefore freeing Rel/NF- $\kappa$ B dimers that become able to translocate to the nucleus and activate the transcription of their target genes. I $\kappa$ B phosphorylation is under the control of high molecular weight complexes with I $\kappa$ B kinase (IKK) activity. The best characterized of these complexes, the IKK signalosome, is composed of two IKKs, IKK1 and IKK2, and a regulatory protein, NEMO [NF- $\kappa$ B essential modulator (23, 24)]. RelA<sup>-/-</sup>, IKK2<sup>-/-</sup> and NEMO<sup>-/-</sup> embryos die between embryonic day 12.5 and 15, with massive liver apoptosis due to enhanced sensitivity to tumor necrosis factor (TNF)- $\alpha$  toxicity (25–28), demonstrating that Rel/NF- $\kappa$ B factors behave as antiapoptotic factors. In contrast, IKK1<sup>-/-</sup> mouse embryos display multiple developmental defects, including a reduced number of apoptotic cells in the interdigital areas of the limb bud (29). This demonstration of a possible proapoptotic effect of Rel/NF- $\kappa$ B factors corroborates previous investigations showing that the expression of cRel correlates with the occurrence of apoptosis in chicken embryos, particularly in the mesenchyme of interdigital areas and in thymocytes, and that cRel induces massive cell death when overexpressed in bone marrow cells (30, 31). Transgenic mice overexpressing RelA or I $\kappa$ B $\alpha$  specifically in the epidermis show epidermal hypoplasia or hyperplasia, respectively (32), indicating that Rel/NF- $\kappa$ B factors can negatively control proliferation. In NEMO<sup>+/-</sup> embryos, some skin defects develop, which are due to both hyperproliferation and increased apoptosis of keratinocytes (33), suggesting that Rel/NF- $\kappa$ B factors may control both proliferation and apoptosis in the same cells, at the same time. Similarly, the disruption of the gene encoding cRel induces a default of activation of mature B and T lymphocytes in response to numerous mitogenic stimuli, due to both a cell cycle block and elevated activation-induced apoptosis (34, 35). In this last case, the Rel/NF- $\kappa$ B transcription factor seems to have a proproliferative effect instead of an antiproliferative one. cRel also displays an antiapoptotic function redundant with that of RelA because double knockout mice for RelA and cRel die with liver apoptosis 2 days before single RelA-deficient mice (36).

Because the senescent state implies profound modifications in the proliferative and apoptotic potentials of cells, Rel/NF- $\kappa$ B factors are likely to participate in the control of this phenomenon. However, very few studies have formally investigated this point (for review, see Ref. 37). Gel retardation assays or transactivation assays performed with WI38, IMR90, or normal diploid fibroblasts derived from foreskin or

Received 1/6/03; revised 10/17/03; accepted 11/6/03.

**Grant support:** Grants from the Centre National de la Recherche Scientifique, Universities Lille 1 and Lille 2, the Association pour la Recherche sur le Cancer, the Ligue contre le Cancer (Comité du Nord), the Institut Pasteur de Lille, the Conseil Régional Nord/Pas-de-Calais, and the European Regional Development Fund. K. Gosselin has a fellowship from the Institut Pasteur de Lille and the Région Nord/Pas-de-Calais. C. Abbadie is Maître de Conférences at the Université Lille 1.

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**Notes:** D. Bernard and K. Gosselin contributed equally to this work. Present address: D. Bernard, Free University of Brussels, Laboratory of Molecular Virology, Faculty of Medicine CP614, 808 route de Lennik, 1070 Brussels, Belgium.

**Requests for reprints:** Corinne Abbadie, UMR 8117, Institut de Biologie de Lille, 1 rue Calmette, BP 447, 59021 Lille Cedex, France. Phone: 33-3-20-87-11-02; Fax: 33-3-20-87-11-11; E-mail: corinne.abbadie@ibl.fr.

oral mucosa indicate that Rel/NF- $\kappa$ B activity and Sp1 and AP1 activities either do not change or decrease during senescence (38–42). In contrast, TNF- $\alpha$ -inducible Rel/NF- $\kappa$ B activity was shown to increase in senescent smooth muscle cells, due to much faster and more extensive I $\kappa$ B $\alpha$  degradation than in young cells (43). To our knowledge, no such investigations were performed with normal primary epithelial cells. However, our previous studies on the function of cRel in HeLa epithelial transformed cells have indicated that it induces proliferation blockage, resistance to TNF- $\alpha$ -induced apoptosis, polynucleation, and lipofuscin accumulation via the up-regulation of manganese superoxide dismutase (MnSOD) and the generation of ROS (44, 45), *i.e.*, biological effects and mechanisms reminiscent of cellular senescence. Our aim in this study was to formally establish the involvement of Rel/NF- $\kappa$ B transcription factors in cellular senescence by using primary epithelial cells that normally senesce *in vitro* (normal human keratinocytes). We demonstrate that (a) overexpressing cRel in young keratinocytes induces a premature senescent phenotype, (b) the endogenous Rel/NF- $\kappa$ B activity increases during normal keratinocyte senescence, (c) reducing this Rel/NF- $\kappa$ B activity with pharmacological inhibitors decreases the appearance of the SA- $\beta$ -Gal marker, (d) both cRel-induced premature senescence and normal senescence are accompanied by an increase in MnSOD expression, and (e) the occurrence of both cRel-induced premature senescence and normal senescence relies on the accumulation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).

## MATERIALS AND METHODS

**Cell Culture and Reagents.** Normal human epithelial keratinocytes (NHEKs), purchased from Clonetics (CC-2501), were obtained from three different donors, all females, but of different races and different ages (Caucasian, 65 years old; black, 58 years old; black, 33 years old). They were grown at 37°C in an atmosphere of 5% CO<sub>2</sub> in KGM-2 BulletKit medium consisting of modified MCBD 153 with 0.15 mM calcium and supplemented with bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrin (CC-3107; Clonetics). Such a serum-free, low-calcium medium was shown to minimize keratinocyte terminal differentiation (46). Cells were seeded as recommended by the supplier and passaged at 70% confluence. The number of population doublings (PDs) was calculated at each passage by using the following equation: PD = ln(number of collected cells/number of plated cells)/ln2. Catalase and gliotoxin were purchased from Calbiochem, and sulfasalazine was purchased from Sigma.

**Adenoviral Vectors.** The open reading frame part of the human *c-rel* cDNA (47) was amplified by PCR using the High Fidelity PCR Master kit (Roche) according to the manufacturer's recommendations with the following oligonucleotides: RelForward, 5'-AAGCTTACCATGGCCTCCGGTGCGTATAAC-3'; and RelReverse, 5'-GATCTCTAGATTACTTGAATAAATTCATATGGAAAGG-3'. The amplification product was inserted into the pAdCMV2 vector. Recombinant adenovirus vectors (AdRel) were obtained by homologous recombination in *Escherichia coli* as described in Ref. 48 (details are available on request). Viral stocks were then created as described in Ref. 49. The generation of a recombinant adenovirus encoding green fluorescence protein (AdGFP) was described in Ref. 50. Viral titers were determined by a plaque assay on 293 cells and defined as plaque-forming units/ml. Cells were infected by adding virus stocks directly to the culture medium at an input multiplicity of 100 viral particles/cell.

**Western Blotting.** Infected cells were lysed directly in the SDS-PAGE loading buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.02% bromophenol blue, and 10%  $\beta$ -mercaptoethanol]. Noninfected cells were lysed in a solution of 27.5 mM HEPES (pH 7.6), 1.1 M urea, 0.33 M NaCl, 0.1 M EGTA, 2 mM EDTA, 60 mM KCl, 1 mM DTT, and 1.1% NP40, and the total protein concentration was measured with the Bio-Rad protein assay. Nuclear extracts were done as described for EMSA analysis. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C extra; Amersham). Equal loading was verified after a Ponceau Red coloration of the membranes. Primary antibodies used were antihuman cRel mouse IgG1 (sc-

6955; Santa Cruz Biotechnology), antihuman I $\kappa$ B $\alpha$  mouse IgG1 (sc-1643; Santa Cruz Biotechnology), antihuman histone H2A rabbit IgG (sc-10807; Santa Cruz Biotechnology), antihuman MnSOD sheep IgG (Calbiochem), antihuman Cu/Zn superoxide dismutase (Cu/ZnSOD) sheep IgG (Calbiochem), antihuman catalase sheep immunoglobulin (The Binding Site), antihuman glutathione peroxidase (GPX) sheep immunoglobulin (The Binding Site), and antihuman actin goat IgG (sc-1616; Santa Cruz Biotechnology). Secondary antibodies used were a peroxidase-conjugated rabbit antiship IgG (Jackson ImmunoResearch Laboratories), a peroxidase-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories), or a peroxidase-conjugated donkey antirabbit IgG. Peroxidase activity was revealed using an enhanced chemiluminescence kit (Amersham).

**Immunofluorescence.** Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. Cells were incubated with antihuman cRel mouse IgG1 (sc-6955; Santa Cruz Biotechnology), washed three times with PBS, and incubated with Rhodamine Red-conjugated antimouse IgG (715-296-150; Jackson ImmunoResearch Laboratories). Nuclei were stained by Hoechst 33258 at 1  $\mu$ g/ml for 3 min.

**Semiquantitative Reverse Transcription-PCR.** Cells were homogenized in Trizol (Gibco-BRL), and total RNAs were isolated according to the manufacturer's recommendations. cDNAs were synthesized using the Gene Amp RNA PCR kit (Perkin-Elmer) and amplified with the gene Amp 9600 PCR system (Perkin-Elmer) in a final volume of 50  $\mu$ l of buffer containing 2.5  $\mu$ l of the retrotranscription product, all four deoxynucleotide triphosphates at 150  $\mu$ M, MgCl<sub>2</sub> (3 mM for cRel, 2 mM for others), 1 unit of Taq gold polymerase (Roche), and each primer at 1  $\mu$ M. Primers used were as follows: cRel forward, AGAGGGGAATGCGTTTATAGATACA; cRel reverse, CAGGGAGAAAAA-CCTGAAAACACA; I $\kappa$ B $\alpha$  forward, CGCCCAAGCACCCGGATACAGC; I $\kappa$ B $\alpha$  reverse, TGGGGTCAGTCACTCGAAGCACAA; and primer for  $\beta$ -actin as described in Ref. 51. Thirty cycles were done at 94°C for 1 min; 53.3°C (cRel), 55°C ( $\beta$ -actin), or 56.9°C (I $\kappa$ B $\alpha$ ) for 1 min; and 72°C for 1 min, with an initial step of 5 min at 95°C. PCR product lengths were 420 (cRel), 503 (I $\kappa$ B $\alpha$ ), and 661 bp ( $\beta$ -actin).

**Bromodeoxyuridine (BrdU) Incorporation Assays, Apoptosis Assays, and SA- $\beta$ -Gal Assays.** To mark proliferating cells, cells were incubated with BrdU (Roche) at 10  $\mu$ M for 6 h. Cells were subsequently fixed and permeabilized as described above and incubated with 40 units/ml DNase I (Promega) and 20 units/ml Exonuclease III (Roche) for 30 min at 37°C. BrdU was revealed by incubations with anti-BrdU mouse IgG (Dako) and Rhodamine Red-conjugated antimouse IgG (715-296-150; Jackson ImmunoResearch Laboratories). Apoptosis was induced by treating cells with recombinant human TNF- $\alpha$  (10 ng/ml; R&D Systems) and cycloheximide (10  $\mu$ g/ml; Sigma) overnight. Cells were fixed as described above. Apoptotic cells were identified by phase-contrast microscopy according to their condensation and their beginning to detach from dish. SA- $\beta$ -Gal assays were done as described in Ref. 17.

**Electrophoretic Mobility Shift Assays.** Nuclear extracts were prepared as described in Ref. 52. Nuclear protein concentrations were measured with the Bio-Rad protein assay. The  $\kappa$ B (5'-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3'), m $\kappa$ B (5'-AGT-TGA-GGC-GAC-TTT-CCC-AGG-C-3'), and Sp1 (5'-ATT-CGA-TCG-GGG-CGG-GGC-GAG-C-3') probes (from Promega or Santa Cruz Biotechnology) were labeled according to the recommendations of Promega and purified using QIAquick Nucleotide Removal kit (28304; Qiagen). Two  $\mu$ g of nuclear extract were incubated with 0.035 pmol of radiolabeled probe according to the recommendations of Promega. Cold competitions were performed by preincubating nuclear extracts with a 100-, 10-, or 1-fold excess of cold  $\kappa$ B or Sp1 probe before the incubation with the radiolabeled probe. For supershift experiments, nuclear extracts were preincubated with 2  $\mu$ l of anti-cRel, anti-RelA, or anti-p50 antibodies (antibodies used were those described in Ref. 53) before the incubation with the radiolabeled  $\kappa$ B probe. DNA-protein complexes were separated from unbound probe by migration on native 4% polyacrylamide gels at 200 V for 2 h.

**Statistics.** *P* calculations were performed with ANOVA (StatView). Differences were considered significant when *P* was <0.05.

## RESULTS

**Overexpression of cRel in Young Primary Keratinocytes.** NHEKs are able to achieve approximately 20 PDs under our culture conditions before they reach the senescence growth plateau and dis-

play the characteristic senescent morphology. The ability of cRel to induce senescence was tested by describing the effects of its overexpression in young NHEKs at 5 PDs and comparing them with the phenotype of senescent NHEKs at 20 PDs. We have constructed a recombinant adenovirus encoding cRel (AdRel) as a vector to overexpress cRel. Cells infected with AdGFP and noninfected cells were used as controls (54).

To check the overexpression of cRel in AdRel-infected young NHEKs, reverse transcription-PCR, immunoblotting, and immunofluorescence experiments were performed 24 h after infection. As shown in Fig. 1A, cRel mRNA and protein were detected in great amounts in AdRel-infected cells. Immunofluorescence experiments revealed cRel overexpression in 70–100% of cells (Fig. 1B). The overexpressed cRel was localized mainly in the nucleus, suggesting that it is transcriptionally active (Fig. 1B).

To further investigate the transcriptional activity of the overexpressed cRel, we first examined the expression of I $\kappa$ B $\alpha$ , a well-established Rel/NF- $\kappa$ B target gene (55, 56). I $\kappa$ B $\alpha$  mRNA and protein accumulated in AdRel-infected cells (Fig. 1A), suggesting that cRel was indeed transcriptionally active. We then checked the ability of the overexpressed cRel to bind DNA. EMSA analyses were performed using nuclear extracts of infected cells 24 and 36 h after infection. The specific Rel/NF- $\kappa$ B DNA binding activity was much higher in AdRel-infected cells than in AdGFP-infected cells at 36 h after infection (Fig. 2A). Supershift experiments revealed that the involved dimers are composed mainly of cRel and p50 (Fig. 2B). This suggests that cRel overexpression increased the expression of p50, which is indeed encoded by a known Rel/NF- $\kappa$ B target gene (57). The up-regulation

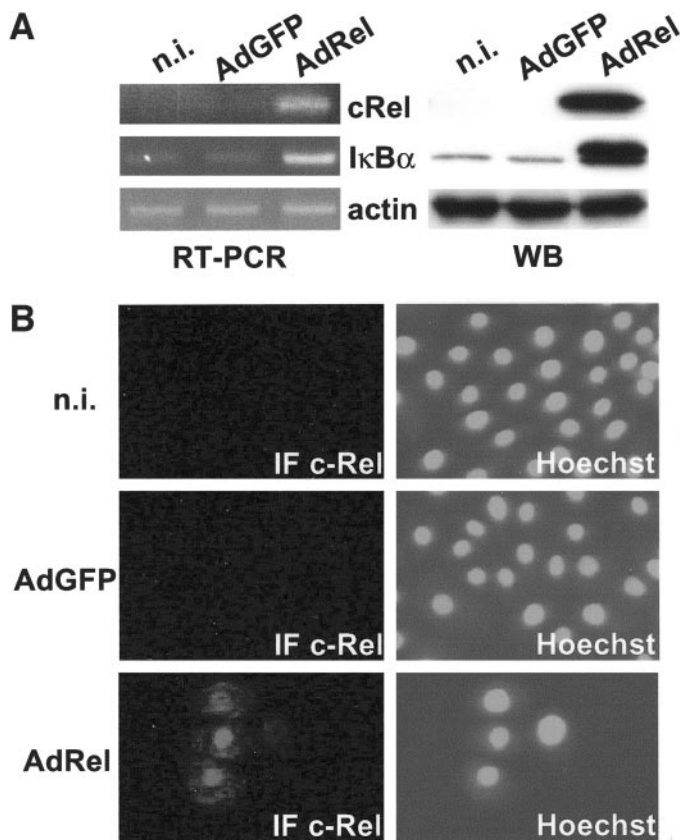


Fig. 1. cRel overexpression in young primary keratinocytes. Young normal human epithelial keratinocytes at 5 PDs were infected with AdGFP or AdRel or not infected (*n.i.*), and 24 h later, the expression of cRel and I $\kappa$ B $\alpha$  was analyzed. A, reverse transcription-PCR. B, Western blot. Actin was used as a loading control. C, immunofluorescence. Nuclei were stained with Hoechst 33258. Notice that cRel-expressing cells are enlarged and that cRel is preferentially located in the nuclei.

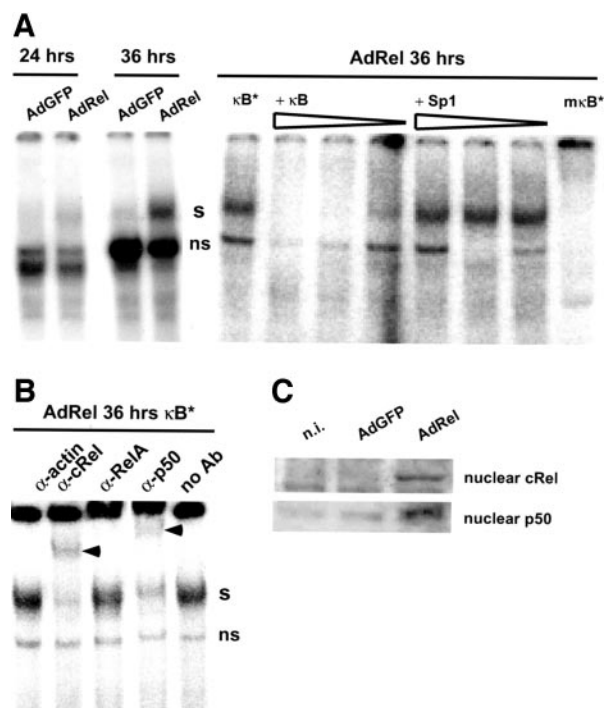


Fig. 2. DNA binding activity of the overexpressed cRel. A, young normal human epithelial keratinocytes at 5 PDs were infected with AdGFP or AdRel, and 24 or 36 h later, nuclear extracts were performed for EMSA analysis. Two  $\mu$ g of each nuclear extract were incubated either with a radiolabeled  $\kappa$ B consensus probe ( $\kappa$ B\*), in the presence or absence of an excess of 100-, 10-, or 1-fold of cold  $\kappa$ B or unrelated Sp1 probe, or with a mutated radiolabeled  $\kappa$ B probe (*mκB\**). *s* indicates the specific band, and *ns* indicates the nonspecific one. B, supershift experiments. Nuclear extracts of AdRel-infected cells 36 h after infection were incubated with the radiolabeled  $\kappa$ B consensus probe in the presence of antibodies directed against cRel, RelA, p50, or actin. Arrowheads indicate the supershifted bands. C, detection of cRel and p50 by Western blot analysis in nuclear extracts of noninfected (*n.i.*), AdGFP-infected, or AdRel-infected cells, 36 h after infection.

and nuclear localization of p50 together with cRel in AdRel-infected cells were confirmed by Western blot analysis performed with nuclear extracts (Fig. 2C).

**Young Keratinocytes Overexpressing cRel Acquire a Flattened Morphology, Stop Proliferating, Resist TNF- $\alpha$ -Induced Apoptosis, and Display SA- $\beta$ -Gal-Activity.** We first evaluated by phase-contrast microscopy the effects of cRel overexpression on the morphology of young NHEKs at 5 PDs and compared it with the morphology of senescent NHEKs at 20 PDs. Forty-eight h after infection, numerous young AdRel-infected cells were enlarged and flattened, compared with young AdGFP-infected cells or young noninfected cells (Fig. 3A). Many of the young AdRel-infected cells contained granules and vacuole-like structures around the nucleus, and some displayed two or three nuclei (Fig. 3B). These features were similarly observed in senescent NHEKs (Fig. 3, A and B).

To examine whether cRel could reproduce a proliferation blockage similar to that of senescent cells, we performed a BrdU incorporation assay on senescent NHEKs at 20 PDs and on young infected NHEKs at 5 PDs, 48 h after infection with AdRel or AdGFP. The results show that the proliferation rate of senescent keratinocytes is about one-third that of young keratinocytes and that the proliferation rate of young AdRel-infected keratinocytes is about one-half that of AdGFP-infected cells. A statistical analysis indicates that these differences are significant, whereas the difference between the proliferation rate of senescent cells and that of young AdRel-infected cells is not (Fig. 4A).

We also compared the apoptosis resistance of young NHEKs overexpressing cRel with that of normal noninfected senescent cells. Apoptosis was induced by an 18-h treatment with TNF- $\alpha$  in the presence of the protein synthesis inhibitor cycloheximide. This treat-

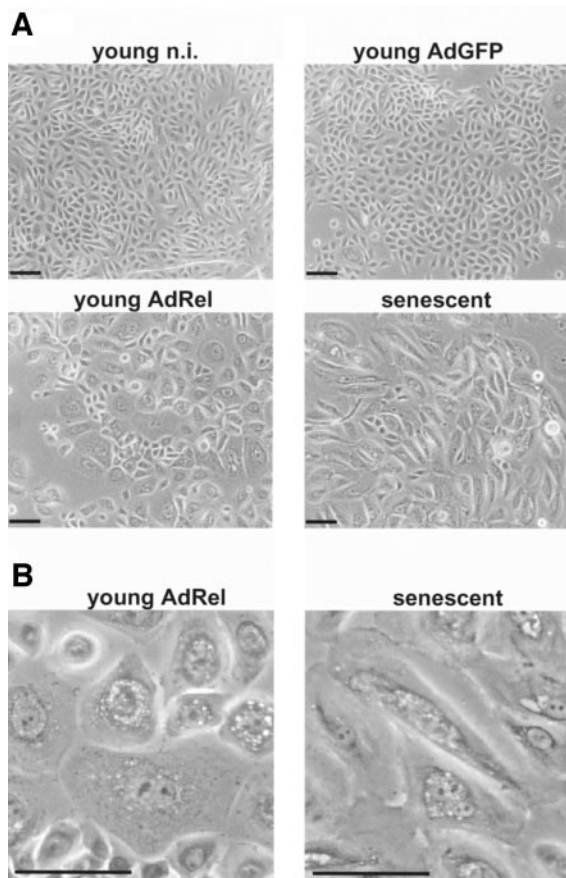


Fig. 3. Morphological comparison of young AdRel-infected keratinocytes and senescent keratinocytes. Young normal human epithelial keratinocytes (5 PDs), noninfected (*n.i.*) or infected with AdGFP or AdRel, and senescent normal human epithelial keratinocytes (20 PDs) were fixed in paraformaldehyde 48 h after infection and observed by phase-contrast microscopy. *A*, numerous enlarged and flattened cells are found in the population of young AdRel-infected cells as well as in senescent cells. *B*, higher magnification of these cells: note granular and vesicular-like material around the nucleus, and the polynucleation in both young AdRel-infected cells and senescent cells. Bars, 100  $\mu$ m.

ment induced the morphological features of apoptosis in 70–80% of young noninfected or AdGFP-infected cells *versus* only 20% of young AdRel-infected cells and only 10% of normal senescent cells. These differences are statistically significant, whereas the difference between the apoptosis rate of young AdRel-infected cells and that of normal senescent cells is not (Fig. 4*B*). It should be noticed that the AdRel-infected young keratinocytes that were still viable after the TNF- $\alpha$  + cycloheximide treatment clearly displayed the morphology of senescent cells (Fig. 4*C*).

SA- $\beta$ -Gal assays, based on the formation of a blue precipitate due to 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside cleavage at pH 6, were performed on young noninfected NHEKs and young NHEKs infected by AdRel and AdGFP, 48 and 72 h after infection, as well as in senescent NHEKs. Numerous large cells that had accumulated the blue precipitate were found in the population of both young AdRel-infected cells and normal senescent cells, whereas young small control cells were negative (Fig. 5).

Therefore, based on morphology, proliferation rate, apoptosis sensitivity, and SA- $\beta$ -Gal activity, we conclude that overexpressing a transcriptionally active member of the Rel/NF- $\kappa$ B family, cRel, provokes premature senescence in young primary keratinocytes.

**Involvement of Rel/NF- $\kappa$ B Activity during Normal Keratinocyte Senescence.** To test the physiological relevance of this effect of cRel overexpression, we first investigated whether the expression level of endogenous cRel changes during normal keratinocyte

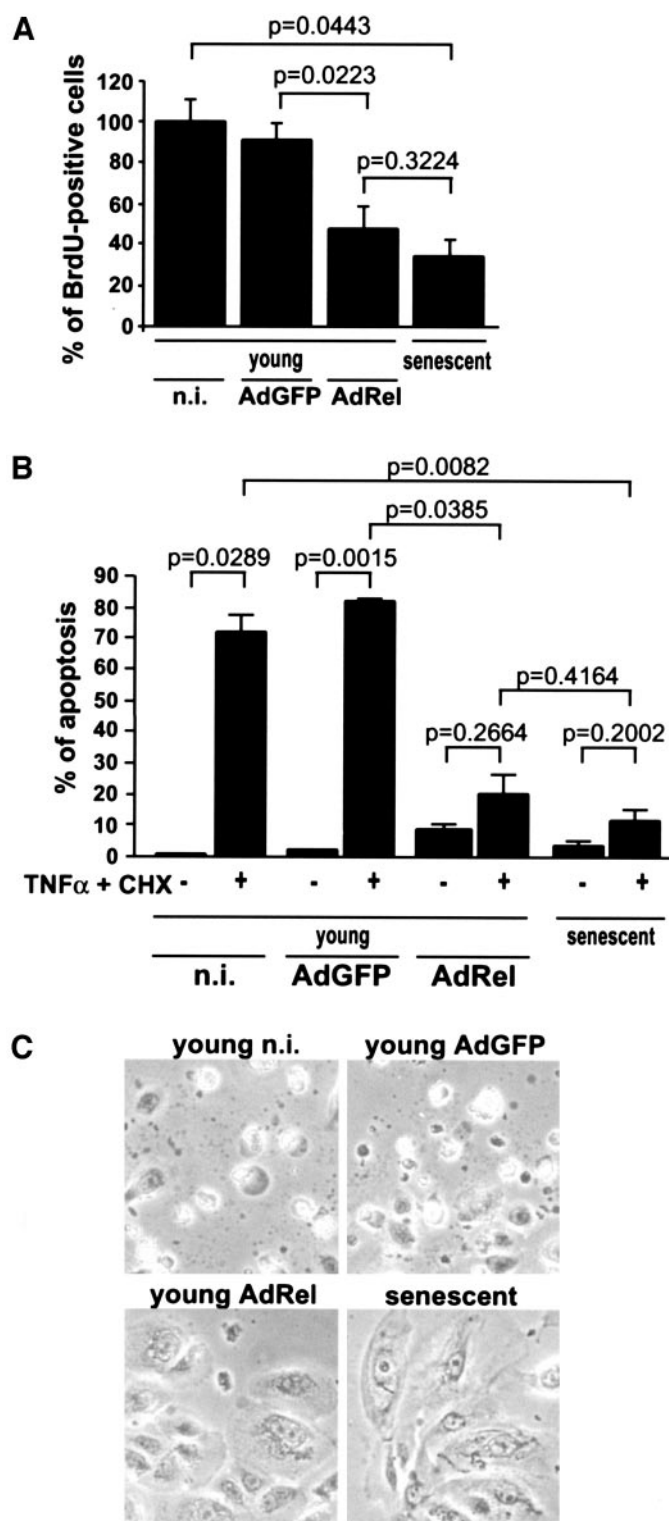


Fig. 4. Replicative capacity and apoptosis resistance of young AdRel-infected keratinocytes and senescent keratinocytes. *A*, 48 h after infection, bromodeoxyuridine incorporation assays were performed on young NHEKs, noninfected (*n.i.*) or infected with AdGFP or AdRel, and senescent NHEKs. Bromodeoxyuridine-positive cells were counted in triplicate, and the percentages were normalized to 100% for young noninfected cells. *B*, young NHEKs, noninfected (*n.i.*) or infected with AdGFP or AdRel, and senescent NHEKs were treated 24 h after infection with TNF- $\alpha$  (10 ng/ml) and cycloheximide (CHX; 10  $\mu$ g/ml) for 18 h. Apoptotic cells were counted in triplicate. *C*, representative pictures of cells treated with TNF- $\alpha$  and cycloheximide.

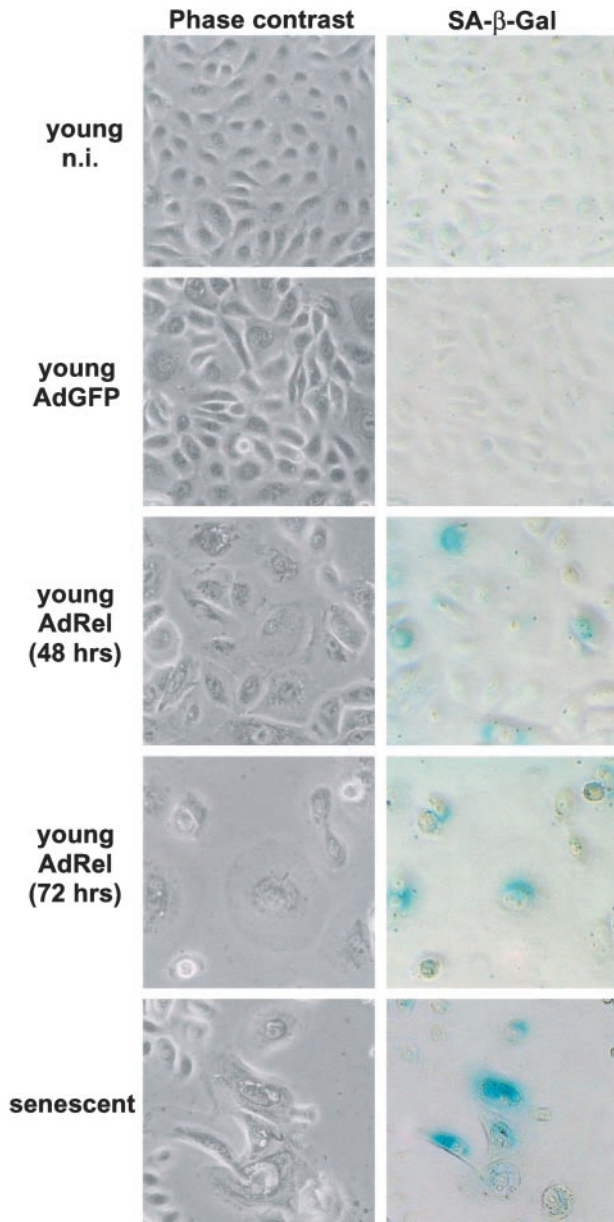


Fig. 5. SA- $\beta$ -Gal activity in young AdRel-infected keratinocytes and senescent keratinocytes. SA- $\beta$ -Gal assays were performed in young NHEKs [noninfected (*n.i.*) or infected with AdGFP or AdRel], 48 and 72 h after infection, or in senescent NHEKs. The accumulation of a blue precipitate, representative of SA- $\beta$ -Gal activity, is observable in senescence-like AdRel-infected cells as well as in normal senescent cells.

senescence. Western blot experiments did not reveal any significant difference in the total amount of cRel protein between young and senescent NHEKs (Fig. 6A). In contrast, the expression level of I $\kappa$ B $\alpha$  greatly increased in senescent cells (Fig. 6A), suggesting an increase in Rel/NF- $\kappa$ B activity. We therefore performed an EMSA analysis. Fig. 6, B and C, shows that the specific Rel/NF- $\kappa$ B DNA binding activity was indeed higher in senescent cells than in young cells. Supershift experiments indicate that the affected dimers were composed of at least cRel, RelA, and p50 (Fig. 6B). Because it was crucial to ensure the equal loading of nuclear extracts of young and senescent cells, we performed with the same nuclear extracts an EMSA analysis of DNA binding on a Sp1 probe and a Western blot analysis of a nuclear protein, histone H2A. Fig. 6C reveals the equal presence of histone H2A in nuclear extracts of young and senescent cells and a decrease in Sp1 binding activity in

senescent cells compared with young cells. In conclusion, these results show that, during normal keratinocyte senescence, there is an increase in Rel/NF- $\kappa$ B, suggesting that Rel/NF- $\kappa$ B factors control, at least in part, the occurrence of senescence.

To investigate the causal link between the increase in Rel/NF- $\kappa$ B activity and the occurrence of senescence, we treated presenescent NHEKs for 5 days with nontoxic doses of sulfasalazine and gliotoxine, two pharmacological inhibitors of Rel/NF- $\kappa$ B activity (58, 59), and we checked for the appearance of SA- $\beta$ -Gal activity. Both inhibitors reduced the percentage of SA- $\beta$ -Gal-positive cells; the decrease induced by sulfasalazine was statistically significant, and that induced by gliotoxine was reproducible but not significant (Fig. 7). We checked the effect of both inhibitors on Rel/NF- $\kappa$ B DNA binding activity by EMSA analysis performed on nuclear extracts. In correlation with SA- $\beta$ -Gal results, only sulfasalazine induced a significant, but not complete, decrease in Rel/NF- $\kappa$ B DNA binding activity (Fig. 7). These results indicate that Rel/NF- $\kappa$ B activity is required for the appearance of the senescent phenotype.

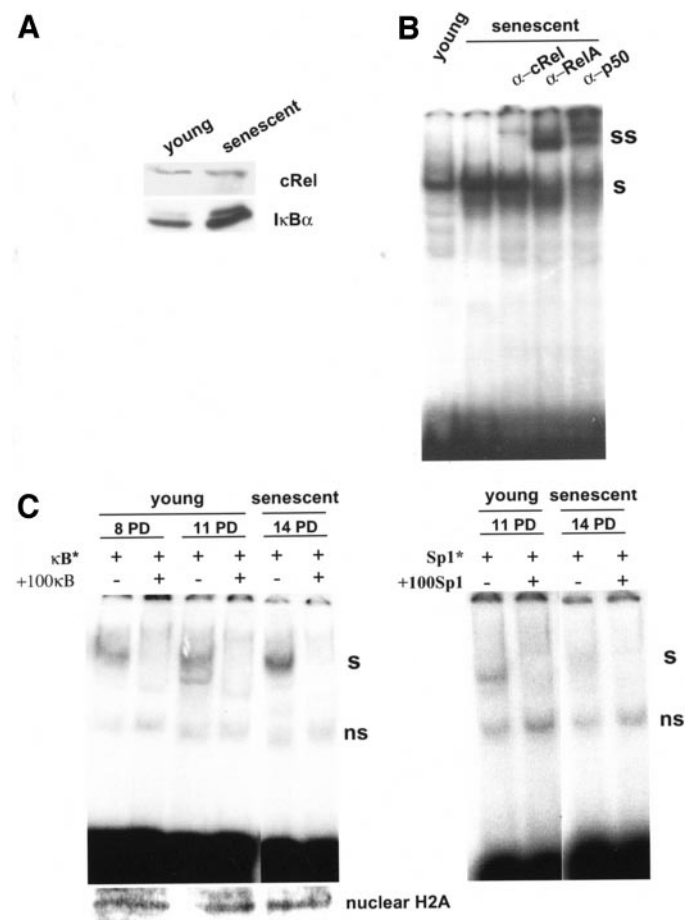


Fig. 6. Comparison of Rel/NF- $\kappa$ B activity in young and senescent keratinocytes. A, Western blot analysis of cRel and I $\kappa$ B $\alpha$  expression in young and senescent NHEKs. B, EMSA analysis of Rel/NF- $\kappa$ B DNA binding activity in young and senescent NHEKs. Nuclear extracts from young or senescent NHEKs were incubated with a radiolabeled  $\kappa$ B consensus probe in the presence or absence of antibodies directed against cRel, RelA, and p50. C, verification of the equal loading of nuclear extracts of young and senescent NHEKs. The same nuclear extracts of young NHEKs at different PDs and senescent NHEKs were used (a) for an EMSA analysis with a radiolabeled  $\kappa$ B consensus probe ( $\kappa$ B\*) in the presence or absence of a 100-fold excess of a cold  $\kappa$ B probe, (b) for an EMSA analysis with a radiolabeled Sp1 consensus probe (Sp1\*) in the presence or absence of a 100-fold excess of a cold Sp1, and (c) for Western blotting analysis of the expression of a nuclear protein, histone H2A. *s* indicates the specific band, *ss* indicates the supershifted bands, and *ns* indicates the nonspecific band.

**Premature cRel-Induced Senescence and Normal Senescence Could Be due to MnSOD Up-Regulation and Ensuing H<sub>2</sub>O<sub>2</sub> Accumulation.** Our previous studies in HeLa epithelial transformed cells have indicated that cRel induces a senescence-like phenotype, *i.e.*, proliferation blockage, resistance to TNF- $\alpha$ -induced apoptosis, polynucleation, and lipofuscin accumulation via the up-regulation of MnSOD and the generation of ROS (44, 45). We therefore examined the expression of MnSOD in the context of primary keratinocyte senescence. As shown in Fig. 8A, MnSOD is induced during both cRel-induced premature keratinocyte senescence and normal keratinocyte senescence. MnSOD is a mitochondrial enzyme that participates in a metabolic pathway that eliminates O<sub>2</sub><sup>-</sup> by a two-step mechanism: first, a dismutation of O<sub>2</sub><sup>-</sup> into H<sub>2</sub>O<sub>2</sub> by MnSOD or Cu/ZnSOD; and then a degradation of H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O by catalase or GPX. Because we and others have shown that an imbalance between H<sub>2</sub>O<sub>2</sub>-producing enzymes (MnSOD and Cu/ZnSOD) and H<sub>2</sub>O<sub>2</sub>-degrading enzymes (catalase and GPX) can create an accumulation of H<sub>2</sub>O<sub>2</sub> resulting in proliferation blockage (44, 60, 61), we examined the expression of Cu/ZnSOD, catalase, and GPX in both premature senescent cRel-

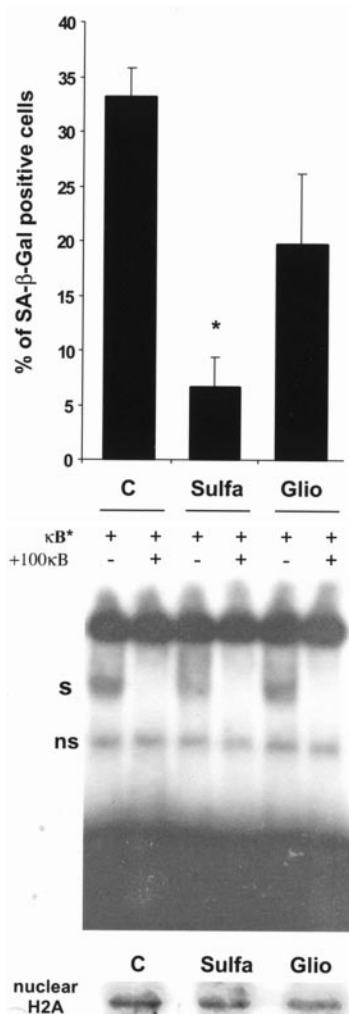


Fig. 7. Requirement of Rel/NF- $\kappa$ B activity for the full occurrence of keratinocyte senescence. Presenescent normal human epithelial keratinocytes at 14 PDs were treated every day for 5 days with 0.5 mM sulfasalazine (*Sulfa*) or 0.05  $\mu$ M gliotoxine (*Glio*) or left untreated (*C*). The second day, nuclear extracts were prepared to check the effect of the pharmacological inhibitors on Rel/NF- $\kappa$ B DNA binding activity. *s* indicates the specific band, and *ns* indicates the nonspecific one. Western blotting analysis of the expression of a nuclear protein, histone H2A, was performed to check the equal loading of the nuclear extracts. On the sixth day, cells were processed for SA- $\beta$ -Gal assays. Each *bar* represents the mean  $\pm$  SD of three points. \*, significant difference. The experiment is representative of two independent ones.

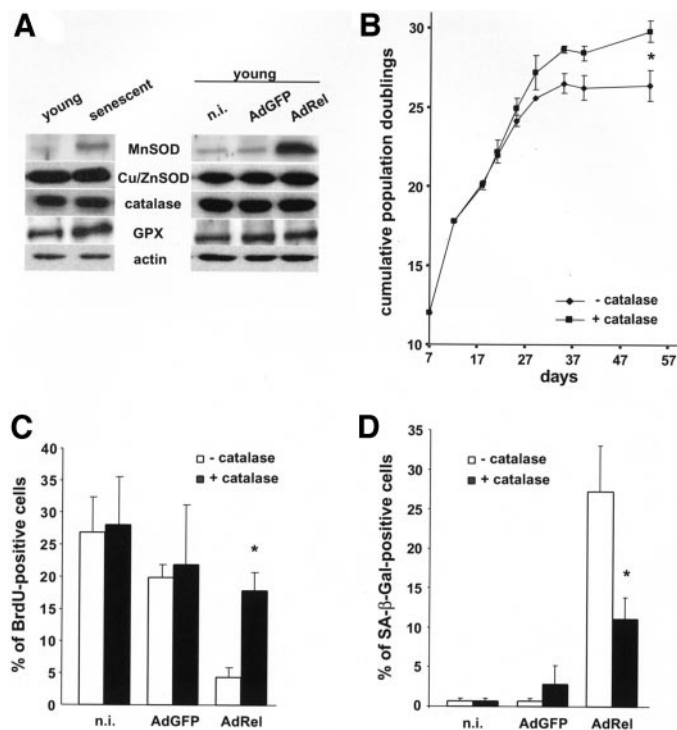


Fig. 8. Role of manganese superoxide dismutase (MnSOD) and H<sub>2</sub>O<sub>2</sub> in premature cRel-induced senescence and in normal senescence. *A*, Western blot analysis of the expression of MnSOD and other enzymes of the O<sub>2</sub><sup>-</sup> degradation pathway in young and senescent NHEKs. *B*, effect of catalase in the occurrence of NHEK senescence. Catalase was added or not added to fresh culture medium at 500 units/ml every 2–3 days. Cells were harvested by trypsinization when they reached 70% confluence in at least one condition, counted in a Coulter counter for population doubling number calculation, and reseeded at equal density for each condition. Each *point* represents the mean  $\pm$  SD of two independent culture dishes. The results are representative of three independent experiments. *C*, effect of catalase in the occurrence of cRel-induced senescence. Young NHEKs were infected with AdGFP or AdRel or not infected (*n.i.*) and treated or not treated every day with 1000 units/ml catalase. Seventy-two h later, cells were processed for bromodeoxyuridine incorporation assay. Bromodeoxyuridine-positive cells were counted in triplicate. The results are representative of two independent experiments. *D*, cells were infected and treated as described above and processed for SA- $\beta$ -Gal activity. SA- $\beta$ -Gal-positive cells were counted in triplicate. \*, significant difference.

expressing cells and normal senescent cells. Fig. 8A shows that the expression of Cu/ZnSOD and catalase did not change between young and senescent cells of both types; expression of GPX increased slightly in senescent cells but did not change in AdRel-infected cells in comparison with AdGFP control cells. Therefore, during both normal and cRel-induced keratinocyte senescence, an imbalance settles in favor of the H<sub>2</sub>O<sub>2</sub>-producing enzyme MnSOD, and hence H<sub>2</sub>O<sub>2</sub> should accumulate.

To establish whether this H<sub>2</sub>O<sub>2</sub> accumulation causes the senescent phenotype, we followed the occurrence of normal and cRel-induced senescence when H<sub>2</sub>O<sub>2</sub> was quenched. Because H<sub>2</sub>O<sub>2</sub> passively diffuses across membranes (62), H<sub>2</sub>O<sub>2</sub> quenching was achieved by adding catalase to the culture medium. Such an addition of catalase to the culture medium of normal keratinocytes delayed the occurrence of the senescence growth plateau from 26 PDs to 29 PDs (Fig. 8B). In addition, in the presence of catalase, both the proliferation blockage and the appearance of SA- $\beta$ -Gal activity induced by cRel overexpression were partly reversed (Fig. 8, *C* and *D*). Therefore, decreasing the H<sub>2</sub>O<sub>2</sub> concentration affects the occurrence of normal and cRel-induced premature senescence, suggesting that H<sub>2</sub>O<sub>2</sub> acts as an effector of the senescent phenotype.

To confirm this role of H<sub>2</sub>O<sub>2</sub>, we examined whether adding H<sub>2</sub>O<sub>2</sub> directly to the culture medium of young keratinocytes would accelerate the occurrence of senescence, as demonstrated previously in

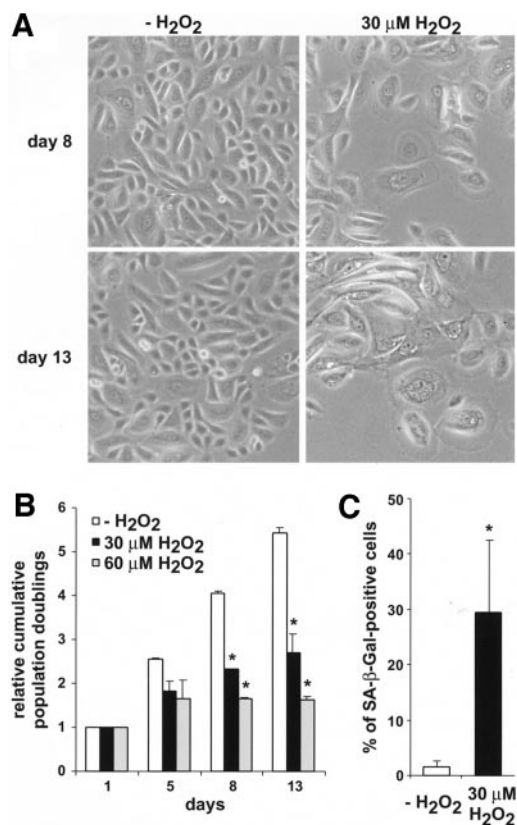


Fig. 9. H<sub>2</sub>O<sub>2</sub> treatment induces a senescence-like state. NHEKs were plated at day 1; treated or not treated with 30 or 60 μM H<sub>2</sub>O<sub>2</sub> for 2 h at day 2, 6, and 9; and analyzed at day 5, 8, and 13. *A*, cell morphologies were examined by phase-contrast microscopy. *B*, cells were harvested by trypsinization, counted in a Coulter counter for PDs number calculation, and reseeded at equal density. *C*, SA-β-Gal assays were performed at the end of the 30 μM treatment. For both histograms, each bar represents the mean ± SD of four independent culture dishes. \*, significant difference.

other cell types [mainly fibroblasts (63–65)], but not in keratinocytes. NHEKs were treated with subtoxic doses of H<sub>2</sub>O<sub>2</sub> (30 or 60 μM) for 2 h, 3 times at 3- or 4-day intervals, and examined for growth rate, morphological changes, and SA-β-Gal activity. As early as the second treatment, cells had acquired a senescent morphology that amplifies with time (Fig. 9A), and the culture ceased to grow (Fig. 9B). At the end of the treatment, numerous cells had become SA-β-Gal positive (Fig. 9C). Therefore, a mild treatment of keratinocytes with H<sub>2</sub>O<sub>2</sub> induces a senescence-like state in a few days.

## DISCUSSION

**Involvement of Rel/NF- $\kappa$ B Transcription Factors in Keratinocyte Senescence.** In this report, we present evidence showing that Rel/NF- $\kappa$ B transcription factors may be involved in keratinocyte senescence. The first evidence is that the forced expression in young keratinocytes of cRel, a transcriptionally active member of the Rel/NF- $\kappa$ B family, induces the main signs of senescence: enlargement and accumulation of granular and vesicular-like material around the nucleus; decrease in replicative capacity; increase in apoptosis resistance; and appearance of SA-β-Gal activity. However, cRel overexpression does not induce telomere shortening (data not shown). We also examined the expression of the cyclin-dependent kinase inhibitors p16<sup>INK4A</sup> and p21, as well as that of p53, which were often reported to be up-regulated during senescence (18). We were unable to detect any increase by Western blot in the amount of p53 and p21 during normal keratinocyte senescence or cRel-induced senescence (data not shown). With regard to p16, in contrast to studies also

performed in human normal keratinocytes (66), our Western blot analyses show a higher amount of p16 in young keratinocytes than in senescent ones (data not shown). We therefore could not consider p16 as a keratinocyte senescence criterion in our hands.

Because senescent keratinocytes and differentiated keratinocytes share some common morphological and molecular features (67), we were concerned about distinguishing cRel effects on senescence from differentiation. Western blot analysis revealed that under our culture conditions, normal senescent cells indeed express the differentiation marker involucrin, but cRel-induced premature senescent cells do not (data not shown). Therefore, the effects of cRel would be restricted to senescence.

The ability of cRel to induce senescence is probably not specific to cRel among the Rel/NF- $\kappa$ B family because the forced expressions of RelA and/or p50 in keratinocytes were shown by others to also induce irreversible growth arrest and SA-β-Gal activity (68). This ability of cRel to induce senescence is also probably not restricted to the keratinocyte cell type because the overexpression of cRel in dermal fibroblasts and mammary epithelial cells also induces a senescence-like phenotype.<sup>3</sup>

The second evidence for the involvement of Rel/NF- $\kappa$ B factors in the control of keratinocyte senescence is that the endogenous constitutive Rel/NF- $\kappa$ B DNA binding activity increases with senescence. This activity relies on dimers composed of at least RelA, cRel, and p50, again suggesting that the involvement of Rel/NF- $\kappa$ B factors in senescence is not restricted to cRel. The increase in Rel/NF- $\kappa$ B activity does not seem to be part of a general senescence-associated increase in transcription factor activity because we detected, in contrast, a decrease in Sp1 activity, as reported previously for fibroblast senescence (39–41). The increase in Rel/NF- $\kappa$ B activity is accompanied and corroborated by the increased expression of two established Rel/NF- $\kappa$ B target genes, I $\kappa$ B $\alpha$  (55, 56) and MnSOD (44, 69, 70).

The last evidence for the involvement of Rel/NF- $\kappa$ B factors in the induction of keratinocyte senescence is that the inhibition of Rel/NF- $\kappa$ B activity in presenescent cells inhibits the appearance of the SA-β-Gal marker. This experiment implied the use of low doses of Rel/NF- $\kappa$ B inhibitors, which are nontoxic on long-term application and do not totally inhibit Rel/NF- $\kappa$ B activity, to avoid any apoptosis induction. The reversal of the senescent phenotype was therefore only partial. The use of more efficient inhibitors such as MG132, BAY11-7082, or the superrepressor form of I $\kappa$ B $\alpha$  was impossible in this assay because they induced massive cell death (data not shown).

**Rel/NF- $\kappa$ B Factors Would Participate in the Occurrence of Senescence by Changing the Redox State of the Cell via the Up-Regulation of MnSOD.** We show here that an up-regulation of MnSOD expression correlates with both cRel-induced premature senescence and spontaneous senescence. The catalytic function of MnSOD is to dismutate the ROS O<sub>2</sub><sup>-</sup> into another ROS, H<sub>2</sub>O<sub>2</sub>. This last one is subsequently degraded in H<sub>2</sub>O by catalase and GPX. We did not observe a coordinated up-regulation of catalase and GPX during either cRel-induced premature senescence or spontaneous senescence. Therefore, H<sub>2</sub>O<sub>2</sub> should accumulate in both situations, hence generating an oxidative stress that could be causal of the senescent phenotype. Supporting this hypothesis, we have shown that quenching H<sub>2</sub>O<sub>2</sub> delays the occurrence of normal and cRel-induced senescence, and, conversely, adding subtoxic doses of H<sub>2</sub>O<sub>2</sub> in the culture medium of young cells induces a premature senescence-like state. The involvement in senescence of SODs and H<sub>2</sub>O<sub>2</sub> has already been documented, mainly in fibroblasts. It has been shown that overexpressing Cu/ZnSOD or directly treating fibroblasts with H<sub>2</sub>O<sub>2</sub> induces a senes-

<sup>3</sup> D. Bernard and K. Gosselin, unpublished data.

cence-like state (60, 63, 65). Conversely, *N-t*-butyl hydroxylamine, which inhibits the formation of  $O_2^-$  and hence that of  $H_2O_2$ , delays senescence of fibroblasts (71). Furthermore, fibroblasts derived from individuals with Down syndrome, who possess a supernumerary copy of the Cu/ZnSOD gene, were shown to senesce earlier than corresponding controls (60). Our results extend the involvement of SODs and  $H_2O_2$  in senescence to keratinocytes and document the control of this mechanism by Rel/NF- $\kappa$ B factors.

**The Apoptosis Resistance of Senescent Cells Could Also Rely on Rel/NF- $\kappa$ B Activity and Up-Regulation of MnSOD.** Senescent cells are apoptosis resistant. This counterintuitive fact was demonstrated for normal human senescent fibroblasts treated with serum withdrawal (13) or DNA-damaging agents [UV radiation, actinomycin D, cisplatin, or ROS (16, 72)]; for senescent T cells treated with dexamethasone, anti-Fas, anti-CD3, galectin-1, IL-2 withdrawal, staurosporine (a protein kinase C inhibitor), or heat shock (15); and for senescent keratinocytes treated with UV radiation (14). In addition, we show here that senescent keratinocytes are resistant to apoptosis induced by TNF- $\alpha$  in the presence of cycloheximide. This is in good agreement with the involvement of Rel/NF- $\kappa$ B factors in senescence because their protective effect against TNF- $\alpha$ -induced apoptosis was largely documented in numerous cell types (73), including keratinocytes (74). MnSOD was also shown to have a protective effect against TNF- $\alpha$ - and serum deprivation-induced apoptosis (75–77) and to participate in the antiapoptotic function of Rel/NF- $\kappa$ B factors (44, 78). The primary function of MnSOD is to eliminate the toxic  $O_2^-$  that is constantly generated in the cell and that can be highly produced in response to some apoptosis inducers such as TNF- $\alpha$  (44, 79, 80), UV radiation (81, 82), cisplatin and actinomycin D (83), anti-Fas (84), and serum deprivation (77). Senescent cells are resistant to apoptosis induced by all these compounds. The up-regulation of MnSOD by Rel/NF- $\kappa$ B factors that we show in this work would account for this property. In all, the up-regulation of MnSOD by Rel/NF- $\kappa$ B factors during senescence would have a dual effect: on one hand, it would make cells more resistant to apoptosis inducers that produce  $O_2^-$ ; but on the other hand, because the  $H_2O_2$ -degrading enzymes are not coordinately up-regulated,  $H_2O_2$  would accumulate, causing some oxidative injuries responsible for the main alterations in senescent cells, *i.e.*, morphological changes, proliferation blockage, and SA- $\beta$ -Gal activity.

**Are Rel/NF- $\kappa$ B Factors Oncogenes or Tumor Suppressor Genes?** Until recently, Rel/NF- $\kappa$ B factor-encoding genes were considered proto-oncogenes. Indeed, *v-rel*, the viral oncogene derived from the avian *c-rel*, is transforming *in vitro* and *in vivo* (85). Human and murine *c-rel* are also transforming *in vitro* and *in vivo*, but after a long delay (86, 87). Chromosomal amplification, rearrangement, or mutations of genes coding for several Rel/NF- $\kappa$ B members are found in many hematopoietic and solid human tumors (88). Constitutive Rel/NF- $\kappa$ B activity was also described in several cancer types, due to either a constitutive activation of the upstream kinases or to mutations in genes encoding I $\kappa$ B proteins (88). As oncogenes, Rel/NF- $\kappa$ B factors would be assumed to allow cells to evade senescence and acquire an immortal and transformed phenotype. However, our results show that Rel/NF- $\kappa$ B factors could, in contrast, be involved in the occurrence of senescence, suggesting that they behave as tumor suppressor genes. In support of this view, targeted expression of I $\kappa$ B $\alpha$  in the epidermis by transgenesis predisposes to the development of spontaneous squamous cell carcinomas (89). Similarly, human reconstituted skin implanted in mouse becomes hyperplastic when it over-expresses I $\kappa$ B $\alpha$  or develops squamous cell carcinoma when it co-expresses I $\kappa$ B $\alpha$  and Ras (90). In addition, it was demonstrated that an immortalized fibroblast cell line derived from a RelA $^{-/-}$  mouse displays numerous properties of a transformed cell line (91). Consid-

ering that Rel/NF- $\kappa$ B factors are often regarded as promising targets in cancer therapy, additional experiments are needed to clarify whether they display oncogenic or tumor suppressor properties.

## ACKNOWLEDGMENTS

We thank N. Rice for the human *c-rel* plasmid; J. Hiscott for the anti-cRel, RelA, and p50 antibodies; and Philippe Becuwe for helpful discussions.

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