Retinoids down-regulate telomerase and telomere length in a pathway distinct from leukemia cell differentiation

Frédéric Pendino*, Maria Flexor*, François Delhommeau[†], Dorothée Buet*, Michel Lanotte*, and Evelyne Ségal-Bendirdjian*[‡]

*Institut National de la Santé et de la Recherche Médicale U496, Centre G. Hayem, Hôpital Saint-Louis, 1, Avenue Claude Vellefaux, 75010 Paris, France; and [†]Institut National de la Santé et de la Recherche Médicale U362, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94800 Villejuif, France

Edited by Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Strasbourg, France, and approved March 20, 2001 (received for review September 29, 2000)

Human telomerase, a cellular reverse transcriptase (hTERT), is a nuclear ribonucleoprotein enzyme complex that catalyzes the synthesis and extension of telomeric DNA. This enzyme is specifically activated in most malignant tumors but is usually inactive in normal somatic cells, suggesting that telomerase plays an important role in cellular immortalization and tumorigenesis. Terminal maturation of tumor cells has been associated with the repression of telomerase activity. Using maturation-sensitive and -resistant NB4 cell lines, we analyzed the pattern of telomerase expression during the therapeutic treatment of acute promyelocytic leukemia (APL) by retinoids. Two pathways leading to the down-regulation of hTERT and telomerase activity were identified. The first pathway results in a rapid down-regulation of telomerase that is associated with retinoic acid receptor (RAR)-dependent maturation of NB4 cells. Furthermore, during NB4 cell maturation, obtained independently of RAR by retinoic X receptor (RXR)-specific agonists (rexinoids), no change in telomerase activity was observed, suggesting that hTERT regulation requires a specific signaling and occurs autonomously. A second pathway of hTERT regulation, identified in the RAR-responsive, maturation-resistant NB4-R1 cell line, results in a down-regulation of telomerase that develops slowly during two weeks of all-*trans* retinoic acid (ATRA) treatment. This pathway leads to telomere shortening, growth arrest, and cell death, all events that are overcome by ectopic expression of hTERT. These findings demonstrate a clear and full dissociation between the process of tumor cell maturation and the regulation of hTERT mRNA expression and telomerase activity by retinoids. We propose telomerase expression as an efficient and selective target of retinoids in the therapy of tumors.

Human telomerase, a ribonucleoprotein enzyme, extends chromosome ends with (TTAGGG)n telomeric sequences, and thus plays a key role in maintaining telomere length and in cellular replicative lifespan (1–3). Several observations indicate that telomeres, DNA-protein structures located at the ends of eukaryotic chromosomes, are important in the immortalization process (4). In most human normal cells, telomeric DNA is progressively lost with each round of cell division (5). Thus, telomeres shorten to a critical length and signal the onset of senescence (6). In contrast, telomere length is stable in immortalized cells, including tumor cells. This stabilization of telomere length seems to be achieved through the induction of a telomerase activity (7). The human telomerase is composed of template RNA components (hTR; ref. 8) and two proteins, telomerase-associated protein-1 (TP-1; ref. 9) and telomerase reverse transcriptase (hTERT), which is thought to be the enzyme's catalytic subunit (10–14). The level of expression of hTERT is the rate-limiting component of this complex: most normal human somatic cells do not have detectable telomerase activity and lack expression of hTERT, whereas most immortalized cells have detectable telomerase activity and express hTERT (10, 11). The correlation between telomerase activity and human tumors has led to the hypothesis that tumor growth requires reactivation of telomerase. Therefore, telomerase is a challenging target for drug development. The factors and mechanisms involved in the regulation of the telomerase remain at primer stages of understanding (15–18). *In vivo* and *ex vivo* observations show that telomerase is repressed during lineage stem cell maturation in embryonic development (19–22). There is mounting evidence that hormones may control telomerase activity (23–30). Indeed, recent results indicate that estrogens (23, 27) and androgens (28, 29) activate telomerase, depending on the target tissues. A possible regulation of hTERT by growth hormone-releasing hormone antagonist (26) or gonadotropin-releasing hormone analogues (30) has also been suggested. Whereas telomerase becomes activated during neoplastic transformation, its activity decreases during differentiation of various immortal cells in response to pharmacological agents, including retinoids (31–37). Repression of telomerase activity was demonstrated to be associated with hTERT mRNA down-regulation, but, noticeably, the expression of hTR and TP1 remained unchanged (11, 37). In these studies, the repression of telomerase has always been considered as a consequence of maturation and/or growth arrest, rather than a direct hormonal regulation.

In this work, we investigated further the biological significance of the action of retinoids in regulating telomerase activity during maturation of tumor cells and questioned whether a mechanistic link between these two phenomena did exist. To this aim, we benefit from a model of acute promyelocytic leukemia (APL), the NB4 cell line (38), where response to retinoic acid (RA) and maturation can be dissociated by several means. This cell line bears the t(15;17) translocation, expresses chimerical promyelocytic leukemia (PML)-retinoic acid receptor (RAR)-α proteins, and is maturation responsive to retinoids (38, 39). Contrastingly, NB4-R1 and NB4-R2 sublines displayed no maturation in the presence of RA (40). However, NB4-R1 cells do respond to RA and become competent to undergo terminal maturation, which can be triggered by cAMP-elevating agents (41), suggesting a crucial interplay between retinoids and cAMP signaling for maturation. No such cooperation exists in the NB4-R2 cells, which were clearly unresponsive to RA, because of the mutation of Gln411 to an in-phase stop codon in the ligand-binding

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: APL, acute promyelocytic leukemia; RA, retinoic acid; ATRA, all-*trans* retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; RAR, retinoic acid receptor; RXR, retinoic X receptor; hTR, human telomerase RNA; hTERT, human telomerase reverse transcriptase; TP-1, telomerase associated protein 1; NBT, nitroblue tetrazolium; TRAP, telomerase repeat amplification protocol; RT-PCR, reverse transcription-PCR; GFP, green fluorescent protein.

[‡]To whom reprint requests should be addressed. E-mail: esegal@jupiter.chu-stlouis.fr.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

 www.pnas.org/cgi/doi/10.1073/pnas.111464998

domain of PML-RARα (42, 43). Although, RAR signaling was disrupted in NB4-R2 cells, retinoic X receptor (RXR)-specific agonists ("rexinoids") cooperate with cAMP signaling to induce maturation of otherwise resistant cells. This novel RARα-independent signaling pathway mediated by "pure" RXR agonists similarly induces maturation of NB4, NB4-R1, and NB4-R2 cells in the context of an activated protein kinase A. Therefore, in this APL model, granulocytic maturation can be induced by two distinct signaling pathways: a RARα-dependent pathway, and a RAR-independent, RXR-dependent pathway (42). Moreover, the NB4-R1 cells exhibit the unique feature of being responsive to, and activated by, RA without embarking on cell maturation. This biological cell system was exploited to determine the consequences of activation of either of each pathway on maturation, cell growth, and telomerase activity, as well as expression of telomerase components (hTERT, hTR, and TP-1). We identified two levels of regulation of telomerase by retinoids and showed that retinoids can regulate telomerase and telomere length independently of cell maturation, leading ultimately to extinction of the tumor cell clone.

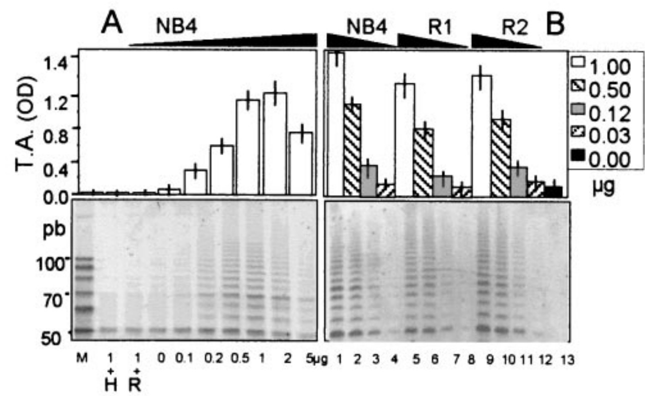

Fig. 1. Telomerase activity in NB4, NB4-R1, and NB4-R2 cells. (*A*) Increasing amounts of NB4 cell extracts were analyzed for telomerase activity (T.A.) by a TRAP-ELISA assay (*Upper*). 1 + R, NB4 extracts (1 μg) pretreated for 20 min at 37°C with RNase A (1 μg/μl). 1 + H, NB4 extracts (1 μg) inactivated by incubation at 85°C for 10 min. The amplified telomerase products obtained in this assay were visualized on electrophoretic gel (*Lower*). (B) Telomerase activity was compared in NB4, NB4-R1, and NB4-R2 cells (*Upper*). The PCR products were visualized on electrophoretic gel (*Lower*). Serial dilutions of each extract were analyzed: 1 μg (lanes 1, 5, and 9); 0.5 μg (lanes 2, 6, and 10); 0.125 μg (lanes 3, 7, and 11); 0.031 μg (lanes 4, 8, and 12); and 0 μg (lane 13). M, molecular weight marker.

Materials and Methods

Reagents and Cell Lines. All-*trans* retinoic acid (ATRA), 9-*cis*-retinoic acid (9-*cis*-RA), and 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP) were purchased from Sigma. Bristol-Myers Squibb generously provided SR11237 (BMS649). The human APL cell line NB4, and its resistant subclones, NB4-R1 and NB4-R2, were cultured as described previously (39–41, 44). Maturation was evaluated by morphology with May-Grünwald-Giemsa and by nitroblue tetrazolium (NBT; Sigma) reduction assay as previously described (40).

Measurement of Telomerase Activity and Telomere Length. Telomerase activity was measured by a modified version of the standard telomerase repeat amplification protocol (TRAP) method (45), the telomerase PCR-ELISA kit (Roche Diagnostics), and TRAPeze Elisa telomerase detection kit (Appligene Quantum) according to the manufacturer's instructions during 22 PCR cycles. Direct visualization of the TRAP ladder is possible on a 15% non-denaturing PAGE, which was stained for 45 min in SYBRGreen I (Roche Diagnostics). Telomere length was measured by using a non-radioactive chemiluminescent assay (Roche Diagnostics) according to the manufacturer's instructions.

PCR Analysis of hTERT, hTR, and TP-1 Expression. Expression of hTERT, hTR, and TP1 mRNA was analyzed by semiquantitative reverse transcription (RT)-PCR amplification. Total cellular RNA was collected from samples by using TRIzol reagent (Life Technologies). RT-PCRs were carried out with 100 ng of mRNA by using the "Reverse Transcription System" (Promega) following the manufacturer's instructions. Two hTERT cDNA amplification protocols were used, and performed as previously described (21). The first hTERT cDNA amplification used TERT-1784S and TERT-1928A primers. β-actin mRNA used as an external standard was amplified from the same cDNA reaction mixture by using the primers 774 and 775. The second hTERT cDNA amplification used TERT-2164S and TERT-2620A oligonucleotides. Primers 774 and 775 were used as above for the β-actin control. hTR cDNA and TP-1 cDNA were amplified by using primers F3b, R3c, and TP1.1, TP1.2, respectively, and conditions described by Nakamura *et al.* (10). Primers K136 and K137 were used for the glyceraldehyde-3-phosphate dehydrogenase control. Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml).

Plasmids and Retroviral Infections. The murine stem cell virus retroviral vector Mig-R1, containing encephalomyocarditis virus internal ribosomal entry sequence and green fluorescent protein (GFP) as a reporter gene (46), was gently provided by W. S. Pear (University of Pennsylvania, Philadelphia, PA). Mig-R1-hTERT vector was obtained by subcloning the catalytic unit of human telomerase (hTERT) sequence from pBABE-puro-hTERT construct (47), which was a generous gift from R. A. Weinberg (Massachusetts Institute of Technology, Cambridge, MA), into Mig-R1 so that 5′ viral long terminal repeat (LTR) promoter drives its expression. Each Mig-R1 construct and pN8 epsilon vectors containing Moloney murine leukemia virus gag pol and vesicular stomatitis virus-G sequences were transfected into the HEK293 EBNA cell line by using Exgen 500 (Euromedex, Souffelweyersheim, France). Mig-R1 control vector and Mig-R1-hTERT supernatants were harvested for further NB4-R1 cell infections, which were carried out in the presence of 4 μg of Polybrene/ml. Infected NB4-R1 cells were sorted two days later for GFP fluorescence.

Results

Constitutive Telomerase Activity in Maturation Responsive and Resistant NB4 Cell Lines. NB4 cells constitutively expressed significant telomerase activity assay as shown in Fig. 1*A Upper*. TRAP assay performed on various quantities of protein extract indicated that 1 μg of protein extract provided a quantitative and reproducible assay for telomerase activity in these cells. Samples with telomerase activity produce a 6-base incremental ladder of TRAP products that can be visualized directly as a TRAP ladder on electrophoretic gels (Fig. 1*A Lower*). A reduced signal intensity of telomerase activity measured by both assays reflected a concentration-dependent response to proteins in the cellular extracts (samples from 1 μg to 0 μg). The signal was not observed when the extracts were pretreated with RNase A or heat to abolish the RNA and protein components of telomerase, respectively. These results confirmed the telomerase specificity of the signal measured by using this assay. In this assay, NB4, NB4-R1, and NB4-R2 cells displayed similar constitutive telomerase activity (Fig. 1*B*).

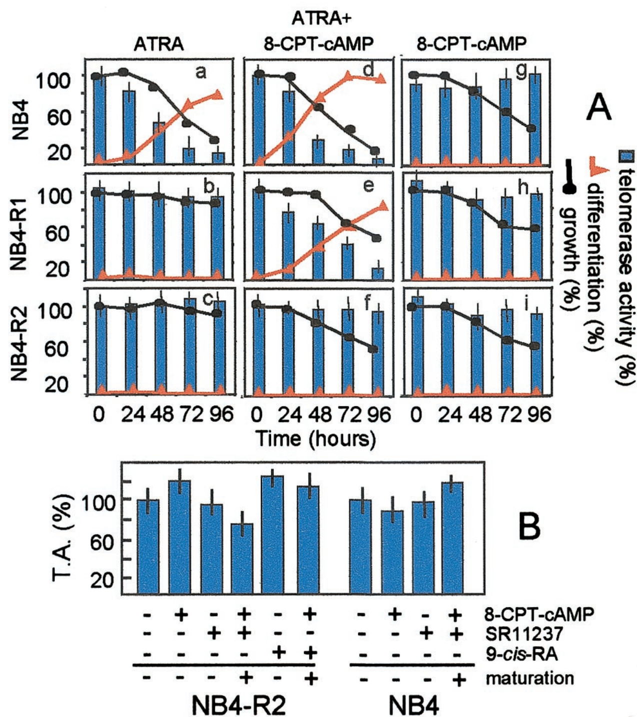

Fig. 2. Telomerase activity in treated NB4, NB4-R1, and NB4-R2 cells. (*A*) Relative telomerase activity (■) in NB4, NB4-R1, and NB4-R2 cells during a 96-h exposure to ATRA (1 μM) alone, 8-CPT-cAMP (100 μM) alone, or a combination of the two. The percentage of cell growth (▲) and differentiation (NBT positive cells; ●) during the same treatment are indicated. (*B*) Telomerase activity (T.A.) in RXR agonist-treated NB4 and NB4-R2 cells. NB4 and NB4-R2 cells were exposed for 96 h to SR11237 (0.2 μM) alone, 8-CPT-cAMP (200 μM) alone, or a combination of the two. NB4-R2 cells were exposed for 96 h to 9-*cis*-RA (0.2 μM) alone, 8-CPT-cAMP (200 μM) alone, or a combination of the two. Telomerase activity was expressed as a percentage of that detected in untreated cells.

Rapid Loss of Telomerase Activity During Retinoid-Induced NB4 Cell Maturation. NB4 cells treated with 1 μM ATRA ceased to grow, became NBT positive (Fig. 2*Aa*), and exhibited morphologic granulocyte-like maturation (not shown). These changes were accompanied by a marked decrease in telomerase activity, to only 10% of the control cell activity after 96 h of treatment. Telomerase activity in extracts from untreated NB4 cells was not inhibited by adding directly 1 μM ATRA to the extract itself (not shown), thus excluding a direct biochemical inhibition of telomerase activity by the drug. Furthermore, when mixed with fixed amounts of untreated NB4 cell extracts, extracts from fully matured NB4 cells did not significantly inhibit the telomerase activity of NB4 cells, thus excluding the induction of a telomerase repressor during the maturation process (not shown). NB4-R1 and NB4-R2 cells, which displayed neither growth inhibition nor maturation during the 96-h exposure to ATRA, showed negligible variations in telomerase activity (Fig. 2 *Ab* and *Ac*). Therefore, the inhibition of telomerase activity correlated with the induction of growth arrest and/or the attainment of a maturated state.

It was previously shown (41) that NB4-R1 cells do respond to RA and become competent to undergo terminal maturation, which can be achieved by cAMP-elevating agents such as 8-CPT-cAMP. No such signaling cooperation was observed in the NB4-R2 cells. Time course experiments showed a down-regulation of telomerase activity by 4 days of treatment, with the combined action of the two drugs in NB4-R1 cells, accompanying cell growth inhibition and granulocytic maturation (Fig. 2*Ae*). The combination of the two signals resulted, as previously shown (41), in an acceleration of the maturation process of NB4 cells, and a faster loss of telomerase activity was observed (Fig. 2*Ad*). In contrast, no significant variation in telomerase activity and no maturation were observed in NB4-R2 cells (Fig. 2*Af*). These results support a relationship between the down-regulation of the telomerase activity and terminal maturation.

Recent observations have suggested that telomerase activity is growth regulated, high in the proliferating cells, and low in quiescent cells (48). As maturated cells ceased growth, the down-regulation of telomerase activity might have been simply related to the cellular proliferation status. However, although on its own 8-CPT-cAMP treatment (100 μM) of NB4 cells was able to induce cell growth inhibition, no change in telomerase activity was observed (Fig. 2 *Ag–Ai*). These results demonstrate that the down-regulation of telomerase activity, which occurred concomitantly with RAR-dependent terminal maturation of APL cells, is not a mere consequence of growth inhibition.

Rexinoid Signaling Does Not Regulate Telomerase Activity in Matured NB4 Cells. The biological actions of RA are mediated by RAR receptors (α,β,γ) and RXR receptors (α,β,γ; ref. 49). Although RAR signaling is disrupted in NB4-R2 cells, a novel RARα-independent-RXR signaling pathway, which requires the simultaneous activation of protein kinase A (through cAMP signaling) can trigger NB4-R2 cell maturation (42). To compare the involvement of each signaling in the regulation of telomerase, telomerase activity was analyzed after treatment of NB4-R2 cells by 8-CPT-cAMP in combination with either a "pure" RXR agonist (SR11237) or the pan-agonist (9-*cis*-RA), which will activate only the RXR pathway in this RAR signaling-defective cell line. Although both SR11237 and 9-*cis*-RA induced full maturation of NB4-R2 cells in the presence of 8-CPT-cAMP (70–90% of NBT-positive cells after 96 h of treatment), through the RXR-signaling pathway, no significant variation in telomerase activity was observed (Fig. 2*B*). Similar results were observed in NB4 cells treated by SR11237 in the presence of 8-CPT-cAMP, whereas it was shown earlier that, in these cells, a rapid telomerase down-regulation accompanied ATRA (RAR-dependent)-induced maturation. Taken together, these results clearly indicate that the down-regulation of telomerase activity associated with maturation of APL cells requires a RAR-signaling pathway and that this regulation is not mechanistically linked to cell maturation, because RXR-dependent maturation occurred in the absence of telomerase down-regulation.

Slowly Developing Loss of Telomerase Activity During a Long-Term ATRA Treatment Leads to Shortening of Telomeres and Cell Death Independently of Cell Maturation. The consequences on cell growth and telomerase expression of a long-term activation of the RAR-dependent pathway were analyzed comparatively in NB4-R1 and NB4-R2 cells cultured for several weeks in the presence of ATRA (1 μM) (Fig. 3*A*).

Within the first passages (4 days) of treatment, telomerase activity of NB4-R1 cells remained at about 85–100% of the untreated controls. From day 5, telomerase activity fell progressively and stabilized at less than 20% of the activity present in the untreated cells by day 11. During the first 2 wk, growth rates were similar in treated and control cultures, with a doubling time of 24–25 h on average. However, in treated cultures, day 14 constituted a turning point where cell growth began to decrease. At day 20, cells stopped dividing and died rapidly without any sign of maturation or senescence (not shown). Importantly, the down-regulation of telomerase activity by ATRA was reversible during the period of incubation preceding this day. Indeed, withdrawal of ATRA from treated NB4-R1 cells at day 11 restored rapidly telomerase activity, and normal growth rate was

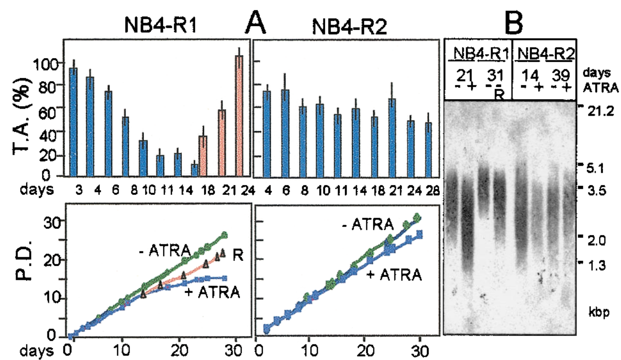

Fig. 3. Change in telomerase activity during a long-term treatment of NB4-R1- and NB4-R2-treated cells. (*A*) NB4-R1 and NB4-R2 were cultured in the continuous presence of ATRA (1 μM). After culturing 11 days in the presence of ATRA, half of the NB4-R1 cell culture was switched to ATRA-free medium and labeled R.A. At the indicated time, protein extracts were prepared and telomerase activity (T.A.) was measured and expressed as a percentage of that detected in untreated cells (*Upper*). Pink histograms refer to the NB4-R1 cells that were cultured 20 days after ATRA removal from the medium. Growth curves showing the cumulative increase in population doubling (P.D.) with time for each culture were presented in the *Bottom* panels. (*B*) Telomere restriction fragment length of NB4-R1 (day 21) and NB4-R2 (days 14 and 39) during a long-term treatment with 1 μM ATRA. Lane R, the NB4-R1 cells that were cultured 20 days after ATRA removal.

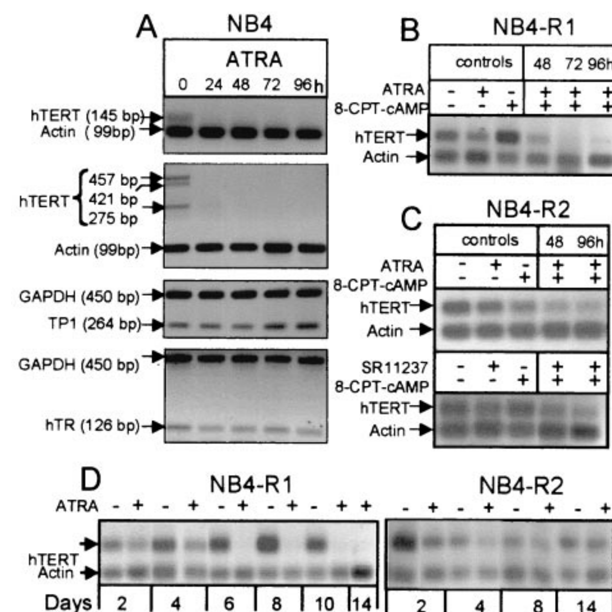

Fig. 4. RT-PCR analysis of expression of hTERT, TP-1, and hTR mRNA in NB4 cell lines. (*A*) NB4 cells were exposed to 1 μM ATRA during 96 h. (*B*) NB4-R1 were exposed to ATRA (1 μM), 8-CPT (200 μM) alone, or to a combination of both drugs. (*C*) NB4-R2 were exposed to ATRA (1 μM), 8-CPT (200 μM) alone, or to a combination of both drugs (*Upper*) or SR11237 (0.2 μM), 8-CPT (200 μM) alone, or to a combination of both drugs (*Lower*). NB4-R1 and NB4-R2 (*D*) cells were cultured in the continuous presence of ATRA (1 μM). Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression were used as controls for RNA loading and RT efficiency.

recovered. In a similar study, in NB4-R2 cells, only a small decrease in growth rate was observed, but cells continued to proliferate, and, after a low initial decrease, telomerase activity remained stable (Fig. 3*A Right*), even after more than 60 days of ATRA treatment (not shown).

The decrease in cell proliferation and cell death likely resulted from a telomerase-dependent mechanism, rather than being caused by a long-term toxicity of the drug. Indeed, the mean telomere length of ATRA-treated NB4-R1 cells was reduced (from ≈3 kb to ≈2 kb) by 21 days of treatment, i.e., after about 10 days of culture with a low level of telomerase activity (Fig. 3*B*). Because NB4-R1 cells have short telomeres, a significant portion of these telomeres is expected to be at critical stage of shortening; thus, even a total erosion of about ≈1 kb in the average telomere length value could be enough to trigger crisis and cell death. Importantly, telomere shortening was reversible. Twenty days after ATRA withdrawal (day 31), the eroded telomeres of ATRA-treated NB4-R1 cells had returned to approximately their initial length. Note that the telomere length of ATRA-treated NB4-R2 cells remained at the same level as the untreated cells throughout at least 60 days of cultivation.

Down-Regulation of hTERT mRNA (Not hTR and TP-1) Expression by Retinoids. To determine the mechanism of action of retinoids on telomerase, we evaluated the expression of all three telomerase components, hTERT, hTR, and TP-1, in NB4, NB4-R1, and NB4-R2 cells after treatment. The treatment of NB4 cells with ATRA (1 μM) led to a rapid down-regulation of hTERT mRNA within 24 h measured by RT-PCR (Fig. 4*A*) using primers either for a region of the transcript upstream of the reverse transcription domain (TERT-1784S and TERT-1928A) or within the reverse transcriptase domain of hTERT (TERT-2164S and TERT-2620A) where two alternate splice sites were previously described (21). In contrast, hTR and TP-1 expression levels of maturated NB4 cells remained largely unchanged, as compared with the control cells. In NB4-R1 cells, the level of hTERT mRNA remained stable during the 96 h of ATRA exposure (Fig. 4*B*). However, treatment of these cells with ATRA and 8-CPT-cAMP induced a rapid decline of hTERT mRNA, closely correlated with telomerase activity and induction of maturation. In contrast, the level of hTERT mRNA in ATRA maturation-resistant NB4-R2-treated cells remained stable during 96 h of ATRA treatment, and the combined signaling of ATRA and 8-CPT-cAMP had no effect. Note that the RXR agonist, SR11237, alone or in combination with 8-CPT-cAMP, also had no effect on hTERT mRNA levels (Fig. 4*C*). These results confirm that the mechanism of telomerase down-regulation observed in maturated NB4 and NB4-R1 cells involves hTERT gene transcription. The down-regulation of telomerase in long-term treatment of NB4-R1 cells with ATRA proceeded through the same mechanism (Fig. 4*D*). As expected, by the high level of telomerase activity, no significant decrease in hTERT expression was observed in the same condition in NB4-R2 cells.

Ectopic Expression of hTERT Durably Protects Long-Term ATRA-Treated NB4-R1 Cells from Cell Death. Altogether, our results suggest that, after a long-term treatment of maturation-resistant NB4-R1 cells with ATRA, loss of telomerase activity and telomere shortening cause cell death. To substantiate this result, we investigated whether ectopic expression of hTERT could rescue long-term ATRA-treated NB4-R1 cells from cell death. For this purpose, NB4-R1 cells infected with either the recombinant virus expressing both hTERT protein and the GFP reporter from the same transcript (designed NB4-R1/hTERT) or the control vector virus expressing only the GFP protein (designed NB4-R1/GFP) were selected on the basis of high GFP expression and sorted.

Cell growth and telomerase expression were then analyzed comparatively in hTERT- and GFP-expressing cell lines during a long-term ATRA treatment. Long-term ATRA treatment of NB4-R1/GFP cells induced a progressive decrease of telomerase activity by day 8. In contrast, NB4-R1/hTERT cells were strongly positive for telomerase activity, and, as expected, this

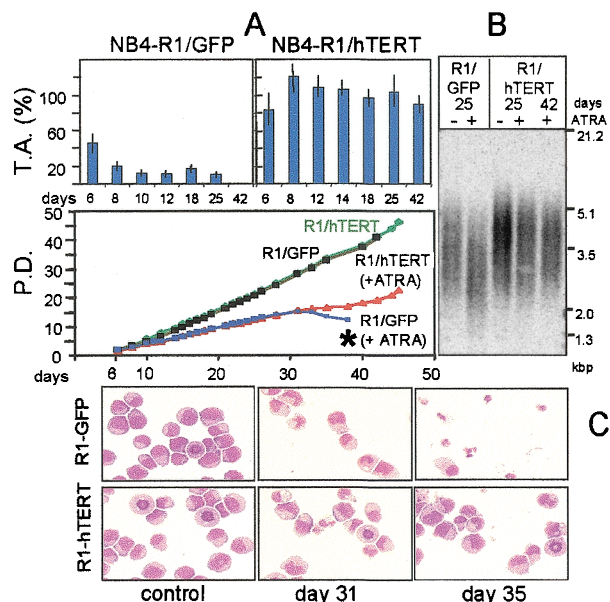

Fig. 5. Change in telomerase activity during a long-term treatment of control NB4-R1/GFP and hTERT-expressing NB4-R1 (NB4-R1/hTERT) cells. NB4-R1/GFP and NB4-R1/hTERT cells were cultured in the continuous presence of ATRA (1 μM) from 3 days after infection. (*A*) At the indicated times, protein extracts were prepared, and telomerase activity was measured. Enzyme activity (T.A.) is expressed as a percentage of that detected in untreated cells (*Upper*). Growth curves showing the cumulative increase in population doubling (P.D.) with time for each culture are presented in the *Bottom* panels. At time *, most of the cells underwent cell death. (*B*) Telomere restriction fragment length of NB4-R1/hTERT (days 25 and 42) and NB4-R1/GFP (days 25 and 42) during a long-term treatment with 1 μM ATRA. (*C*) Differences in growth and morphology of NB4-R1/GFP and NB4-R1/hTERT cells. At day 31, morphologies are similar in both cell culture. However, pictures of mitosis were more frequent in the NB4-R1/hTERT cell culture. At day 35, most of the NB4-R1/GFP cells had undergone cell death. In contrast, the NB4-R1/hTERT cells continued to proliferate, indicating that rescue from cell death and proliferation occurred as a direct result of ectopic expression of hTERT.

activity remained stable during treatment. During the first 4 wk of culture in the continuous presence of ATRA (Fig. 5), both NB4-R1/GFP and NB4-R1/hTERT demonstrated an identical period of reduced growth despite marked differences in the levels of hTERT. This observation indicates that some effects of ATRA on cell growth occurred in a telomerase-independent mechanism. Whereas NB4-R1/GFP cells entered crisis suddenly after day 31 and underwent massive cell death, NB4-R1/hTERT cells continued to proliferate.

The mean telomere length of NB4-R1/GFP cells was reduced at day 25. Even though it was not possible to show a significant increase in telomerase activity in NB4-R1 cells expressing ectopic telomerase, the average telomere length of untreated cells was slightly longer than that of untreated NB4-R1/GFP cells. Furthermore, in these cells, a long-term ATRA treatment failed to induce a significant shortening of telomeric length. Because ectopic expression of hTERT was sufficient to prevent both telomere shortening and cell death, these results clearly demonstrate that repression of telomerase and the subsequent telomere shortening are the main causes of cell death. However, it could not be formally excluded that long-term ATRA treatment activates other cellular processes that cooperate with telomere shortening to trigger cell death.

Finally, the new retroviral-transfected NB4-R1/hTERT cell line exhibited normal granulocytic maturation when cells were treated with a combination of ATRA and 8-CPT-cAMP (not shown). These data are in keeping with the important notion that hTERT steady expression neither prevents, nor alters, granulocytic maturation, whereas hTERT activity remains a key parameter that determines long-term cell survival and thus the fate of tumor cells failing to mature with retinoids.

Discussion

Previous reports have indicated that terminal maturation of immortal cells, including HL60 and NB4 cells, is associated with a rapid inhibition of telomerase activity, preceded by a down-regulation of the telomerase reverse transcriptase expression (31–37). Given that under these conditions the regulation of telomerase occurred without telomere shortening (36), this phenomenon should not contribute to extinguish the tumor cell clone. In addition, it has been reported (32) that, in two murine cell lines (p19 embryonic carcinoma and Neuro2A neuroblastoma cells), maturation with ATRA did not result in a loss of telomerase activity. Therefore, the question as to whether telomerase could be regulated independently of cell maturation, and thus therapeutically exploited in maturation-resistant cells, remained to be clarified.

In the present work, we showed two levels of regulation of telomerase by retinoids in NB4 cells (Table 1). A rapid down-regulation associated with ATRA-induced granulocytic maturation of NB4 cells, and a slowly developing repression during long-term treatment of maturation-resistant cells by ATRA that led to shortening of telomeres and cell death. In both cases, the

Table 1. Effect of retinoids, rexinoid, and 8-CPT-cAMP treatments on telomerase expression, maturation, growth inhibition, and cell death

	NB4, 4 days						NB4-R1						NB4-R2, 4 days					
							4 days				8 days	20 days						
Treatments, days																		
ATRA	−	+	−	+	−	−	−	+	−	+	+	+	−	+	−	+	−	−
SR11237	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	+
cAMP	−	−	+	+	−	+	−	−	+	+	−	−	−	−	+	+	−	+
Biological responses																		
hTERT mRNA	C	↘	=	↘	=	=	C	=	=	↘	↘	↘	C	=	=	=	=	=
Telomerase activity	C	↘	=	↘	=	=	C	=	=	↘	↘	↘	C	=	=	=	=	=
Maturation	NO	**YES**	NO	**YES**	NO	**YES**	NO	NO	NO	**YES**	NO	NO	NO	NO	NO	NO	NO	**YES**
Growth inhibition	NO	**YES**	**YES**	**YES**	NO	**YES**	NO	NO	**YES**	**YES**	NO	**YES**	NO	NO	**YES**	**YES**	NO	**YES**
Cell death	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	**YES**	NO	NO	NO	NO	NO	NO

Variations are estimated compared to untreated cells (C). +, the presence of the corresponding drug in the cell culture; −, its absence; =, no change compared with C; ↘, a strong decrease compared with C. The data correspond to experiments described in Figs. 2, 3, and 4 and have been confirmed by at least three independent experiments.

loss of telomerase activity resulted from a down-regulation of hTERT mRNA, through the activation of a RAR-dependent signaling pathway.

Our findings provide direct evidence, in human cells, of a dissociation between the loss of telomerase activity and the process of maturation. These findings are of importance for the therapy of APL because they show that a telomerase-dependent mechanism can be targeted by retinoids in maturation-resistant cells, provided that the RAR signaling remained functional. The antitumor potential of this biological response comes from telomere shortening and consequent growth arrest and cell death. However, our observation of a regrowth of telomeres to initial length on cessation of ATRA addition and cell recovery furnishes the evidence that any benefit of this antitumor response would be lost if the treatment is prematurely interrupted. Combining maturation therapy and telomerase-targeted therapy, one could envisage the eradication of both maturation-sensitive and -resistant tumor cells, sequentially using the same drug. The integration of a telomerase-based antitumor response in therapeutic protocols should now be considered.

Our results have broader implications. It has been shown (23, 27–29) that hormones, including estrogens and androgens, regulate positively the expression of telomerase in hormone-responsive cell systems. Given their negative action on telomerase activity, retinoids may participate in an equilibrium between pro- and anti-telomerase signaling, necessary to tissue homeostasis during development, in addition to their role as antitumor regulators. The implication of such networks of hormonal signaling for the regulation of telomerase in several important human pathologies should now be investigated further.

We thank Drs. A. Bennaceur-Griscelli, E. Lauret, and V. Schiavon for helpful discussions, retroviral infections, and cell sorting. We are grateful to Dr. R. Weinberg (Massachusetts Institute of Technology) for provision of pBABE-puro-hTERT construct and to Dr. W. S. Pear (University of Pennsylvania) for providing us the murine stem cell virus retroviral vector Mig-R1. We thank Dr. G. Benoit for helpful discussions and Dr. H. Gronemeyer for providing us aliquots of the synthetic RXR agonist, SR11237. This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Ligue Nationale contre le Cancer, and Association pour la Recherche contre le Cancer (ARC nos. 5384, 5707, and 5606). M.F. was supported by Association Claude Bernard.

1. Blackburn, E. H. (1992) *Annu. Rev. Biochem.* **61,** 113–129.
2. Nakamura, T. M. & Cech, T. R. (1998) *Cell* **92,** 587–590.
3. De Lange, T. (1998) *Science* **279,** 334–335.
4. Harley, C. B., Kim, N. W., Prowse, K. R., Weinrich, S. L., Hirsch, K. S., West, M. D., Bachetti, S., Hirte, H. W., Counter, C. M., Greider, C. W., *et al*. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **59,** 307–315.
5. Harley, C. B, Futcher, A. B. & Greider, C. W. (1990) *Nature (London)* **345,** 458–460.
6. Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W. & Harley, C. B. (1992) *Proc. Natl. Acad. Sci.* USA **89,** 10114–10118.
7. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B. & Bachetti, S. (1992) *EMBO J.* **11,** 1921–1929.
8. Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R. Chang, E., Allsoop, R. C., Yu, J., *et al.* (1995) *Science* **269,** 1236–1241.
9. Harrington, L., McPhail, T., Mar, V., Zhou, W., Oulton, R., Amgen EST Program, Bass, M. B., Isabel, A. & Robinson, M. O. (1997) *Science* **275,** 973–977.
10. Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B. & Cech, T. R. (1997) *Science* **277,**955–959.
11. Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R., L., Davidoff, M. J., Liu, Q., *et al*. (1997) *Cell* **90,** 785–795.
12. Kilian, A., Bowtell, D. D., Abud, H. E., Hime, G. R., Venter, D. J., Keese, P. K., Duncan, E. L., Reddel, R. R. & Jefferson, R. A. (1997) *Hum. Mol. Genet.* **6,** 2011–2019.
13. Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, J. B., *et al*. (1998) *Nat. Genet.* **17,** 498–502.
14. Beattie, T. L., Zhou, W., Robinson, M. O. & Harrington, L. (1998) *Curr. Biol.* **8,**177–180.
15. Takakura, M., Satoru, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M. & Inoue, M. (1999) *Cancer Res.* **59,** 551–557.
16. Horikawa, O., Cable, P. L., Afshari, C. & Barrett, J. C. (1999) *Cancer Res.* **59,** 826–830.
17. Greenberg, R. A., O'Hagan, R. C., Deng, H., Xiao, Q., Hann, S. R., Adams, R. R., Lichtsteiner, S., Chin, L., Morin, G. B. & DePinho, R. A. (1999) *Oncogene* **18,** 1219–1226.
18. Gunes, C., Lichtsteiner, S., Vasserot, A. P. & Englert, C. (2000) *Cancer Res.* **60,** 2116–2121.
19. Martin-Riviera, L., Herrera, E., Albar, J. P. & Blasco, M. *A.* (1998) *Proc. Natl. Acad. Sci.* USA **95,** 10471–10476.
20. Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W. & Shay, J. W. (1996) *Dev. Genet.* **18,** 173–179.
21. Ulaner, G. A., Hu, J.-F., Vu, T. H., Giudice, L. C. & Hoffman, A. R. (1998) *Cancer Res.* **58,** 4168–4172.
22. Yamaguschi, Y., Nozawa, K., Savoysky, E., Hayakawa, N., Nimura, Y. & Yoshida, S. (1998) *Exp. Cell Res.* **242,** 120–127.
23. Kyo, S., Takakura, M., Kanaya, T., Zhuo, W., Fujimoto, K., Nishio, Y., Orimo, A. & Inoue, M. (1999) *Cancer Res.* **59,** 5917–5921.
24. Tanaka, M., Kyo, S., Takakura, M., Kanaya, T., Sagawa, T., Yamashita, K., Okada, Y., Hiyama, E. & Inoue, M. (1998) *Am. J. Pathol.* **153,** 1985–1991.
25. Bednarek, A. J., Chu, Y. & Albaz, C. M. (1998) *Oncogene* **16,** 381–385.
26. Kiaris, H. & Schally, A. V. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 226–231.
27. Misiti, S., Nanni, S., Fontemaggi, G., Cong, Y.-S., Wen, J., Hirte, H. W., Piaggio, G., Sacchi, A., Pontecorvi, A., Bachetti, S. & Farsetti, A. (2000) *Mol. Cell. Biol.* **20,** 3764–3771.
28. Meeker, A. K., Sommerfeld, H. J. & Coffey, D.S. (1996) *Endocrinology* **137,** 5743–5746.
29. Soda, H., Raymond, E., Sharma, S., Lawrence, R., Davidson, K., Oka, M., Kohno, S., Izbicka, E. & Von Hoff, D. D. (2000) *Prostate* **43,** 161–168.
30. Ohta, H., Sakamato, H. & Satoh, K. (1998) *Cancer Lett.* **134,** 111–118.
31. Sharma, H. W., Sokoloski, J. A., Perez, J. R., Maltese, J.-Y., Sartorelli, A. C., Stein, C. A., Nichols, G., Khaled, Z., Telang, N. T. & Narayanan, R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 12343–12346.
32. Bestilny, L. J, Brown, C. B, Miura, Y., Robertson, L. D. & Riabowol, K. *T.* (1996) *Cancer Res.* **56,** 3796–3802.
33. Xu, D., Gruber, A., Peterson, C. & Pisa, P. (1996) *Leukemia* **10,** 1354–1357.
34. Albanell, J., Wan, W., Mellado, B., Guanawardane, R., Scher, H. I., Dmitrovsky, E. & Moore, M. A. S. (1996) *Cancer Res.* **56,** 1503–1508.
35. Nason-Burchenal, K., Maerz, W., Albanell, J., Allopenna, J., Martin, P., Moore, M. A. S. & Dmitrovsky, E. (1997) *Differentiation* **61,** 321–331.
36. Yamada, O., Takanashi, M., Ujihara, M. & Mizoguchi, H. (1998) *Leuk. Res.* **22,** 711–717.
37. Xu, D., Gruber, A., Björkholm, M., Peterson, C. & Pisa, P. (1999) *Br. J. Cancer* **80,** 1156–1161.
38. Lanotte, M., Martin-Thouvenin, V., Najman, S., Ballerini, P., Valensi, F. & Berger, R. (1991) *Blood* **77,** 1080–1086.
39. De Thé, H., Chomienne, C., Lanotte, M., Degos, L. & Dejean, A. (1990) *Nature (London)* **347,** 558–561.
40. Duprez, E., Ruchaud, S., Houge, G., Martin-Thouvenin, V., Valensi, F., Kastner, P., Berger, R. & Lanotte, M. (1992) *Leukemia* **6,** 1281–1287.
41. Ruchaud, S., Duprez, E., Gendron, M. C., Houge, G., Genieser, H. G., Jastorff, B., Doskeland, S. O. & Lanotte, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8428–8432.
42. Benoit, G., Altucci, L., Flexor, M., Ruchaud, S., Lillehaug, J., Raffelsberger, W., Gronemeyer, H. & Lanotte, M. (1999) *EMBO J.* **18,** 7011–7018.
43. Duprez, E., Benoit, G., Flexor, M., Lillehaug, J. R. & Lanotte, M. (2000) *Leukemia* **14,** 255–261.
44. Bruel, A., Benoit, G., De Nay, D. & Lanotte, M. (1995) *Leukemia* **9,** 1173–1184.
45. Kim, N., Ma, M. P., Prowse, K., Harley, C., West, M., Ho, P., Coviello, G., Wright, W., Weinrich, S. & Shay, J. (1994) *Science* **266,** 2011–2015.
46. Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karnell, F. G., Bakkour, S., Lee, J. Y., Kadesch, T., Hardy, R. R., Aster, J. C. & Pear, W. S. (1999) *Immunity* **11,** 299–308.
47. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W. & Weinberg, R.A. (1999) *Nature (London)* **400,** 464–468.
48. Greider, C. W. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 90–92.
49. Chambon, P. (1996) *FASEB J.* **10,** 940–954.