Oncogene (1998) 16, 1217–1222

SHORT REPORT

Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase

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The expression of telomerase, the enzyme that synthesizes telomeric DNA *de novo*, is suppressed in normal somatic human cells but is reactivated during tumorigenesis. This reactivation appears to arrest the normal loss of telomeric DNA incurred as human cells divide. Since continual loss of telomeric DNA is predicted to eventually limit cell proliferation, activation of telomerase in cancer cells may represent an important step in the acquisition of the cell immortalization which occurs during tumor progression. The telomerase holoenzyme is composed of both RNA and protein subunits. In humans, mRNA expression of *hTERT* (*hEST2*), the candidate telomerase catalytic subunit gene, appears to parallel the levels of telomerase enzyme activity, suggesting that induction of *hTERT* is necessary and perhaps sufficient for expression of telomerase activity in tumor cells. To test this model directly, we ectopically expressed an epitope-tagged version of *hTERT* in telomerase-negative cells and show that telomerase activity was induced to levels comparable to those seen in immortal telomerase-positive cells and that the expressed hTERT protein was physically associated with the cellular telomerase activity. We conclude that synthesis of the hTERT telomerase subunit represents the rate-limiting determinant of telomerase activity in these cells and that this protein, once expressed, becomes part of the functional telomerase holoenzyme.

Keywords: telomerase; *hTERT*; *hEST2*; cellular immortalization

Most normal human somatic cells lack active telomerase, the enzyme that synthesizes telomeric DNA *de novo*. For this reason, their chromosomes undergo a loss of telomeric DNA with every round of DNA replication (Harley *et al*., 1990, 1994; Hastie *et al*., 1990). When normal human somatic cells are driven to continue to divide inappropriately by transformation with viral oncogenes, they eventually reach crisis, at which point telomeres become extremely short and most cells die. In those rare cells that manage to overcome crisis and continue to divide indefinitely, telomere shortening is arrested either by the activation of telomerase (Counter *et al*., 1992; Harley *et al*., 1994) or, less often, by a telomerase-independent mechanism of maintaining telomere length (Murnane *et al*., 1994; Bryan *et al*., 1995).

Cells from primary human tumors, unlike normal human somatic tissues, can often be established as permanent cell lines. Strikingly, the vast majority of both primary tumors and cancer-derived cell lines are telomerase-positive (Counter *et al*., 1994; Harley *et al*., 1994; Kim *et al*., 1994). The simplest interpretation of these data is that the process of telomere shortening ultimately limits the lifespan of human cells, and that this proliferative barrier is surmounted through the illegitimate activation of telomerase, or, in some cases, by telomerase-independent mechanisms. Thus, the activation of telomerase may represent an important step for the continued survival of cancer cell lineages.

To investigate the mechanism by which telomerase is activated in human cancers, we and others have begun efforts to clone the genes encoding the components of human telomerase. The telomerase enzyme is a ribonucleoprotein complex (Greider and Blackburn, 1985, 1987; Greider, 1995). In humans, the *hTR* gene encodes the RNA component of telomerase (Feng *et al*., 1995), whereas the *TP1*/*TLP1* gene encodes a telomerase-associated protein of unknown function (Harrington *et al*., 1997; Nakayama *et al*., 1997). The expression of these components does not always correlate with telomerase activity (Feng *et al*., 1995; Avilion *et al*., 1996; Harrington *et al*., 1997; Nakayama *et al*., 1997) and therefore does not appear to be rate-limiting for telomerase activation.

We and others have cloned a human cDNA that encodes the putative telomerase catalytic protein subunit (Meyerson *et al*., 1997; Nakamura *et al*., 1997). This protein, which was originally named hEST2 (Meyerson *et al*., 1997) or hTRT (Nakamura *et al*., 1997) has now been renamed hTERT, according to the HUGO Nomenclature Committee of the Genome Database. hTERT shares significant sequence similarity with the catalytic subunit of telomerases from the budding yeast *Saccharomyces cerevisiae* (*EST2*) (Lendvay *et al*., 1996; Counter *et al*., 1997; Lingner *et al*., 1997), the fission yeast *Schizosaccharomyces pombe* (*TRT1*) (Nakamura *et al*., 1997) and the ciliate *Euplotes aediculatus* (p123) (Lingner *et al*., 1997). *hTERT* mRNA is detected in telomerase-positive tissues, cancer cell lines and tumors, but not in cells or tissues known to lack enzymatic activity (Kilian *et al*., 1997; Meyerson *et al*., 1997; Nakamura *et al*., 1997). In addition, *hTERT* mRNA expression is

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Received 26 November 1997; revised 2 January 1998; accepted 3 January 1998

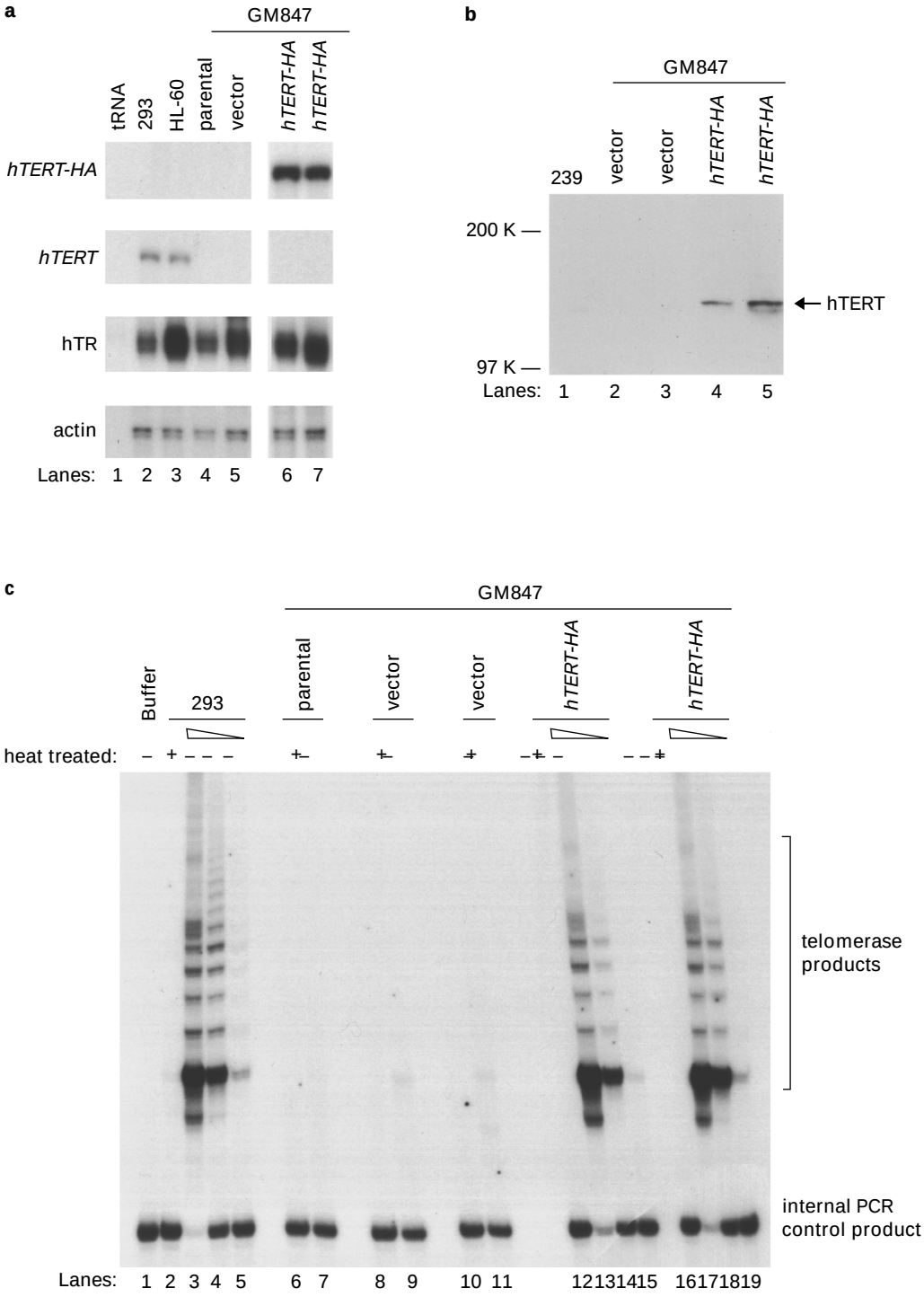

Figure 1 Induction of telomerase activity by stable expression of *hTERT* in telomerase-negative GM847 cells. (**a**) Expression of *hTERT-HA* mRNA in transfected cells. Two copies of the HA epitope tag were introduced in frame at the 3′ end of the *hTERT* cDNA, which was subsequently cloned into the vector pCI-neo (Promega, Madison, WI), yielding the plasmid pCI-neo-*hTERT-HA* (Genbank accession #AF043739). This construct or the pCI-neo vector was transfected into the cell line GM847 using the Superfect reagent (Qiagen, Chatsworth, CA), and stable transfected clones were selected for on 500 μg/ml of G418. RNA was isolated from positive control 293 and HL60 cells which are known to express high levels of telomerase activity (Meyerson *et al*., 1997), the parental line GM847 and either one or two independent GM847 sublines transfected with either pCI-neo or pCI-neo-*hTERT-HA*, respectively. 20 μg of RNA from these cells were hybridized with the appropriate radiolabeled RNA probes, digested with RNases A and T1 and resolved and analysed on 6% polyacrylamide gels, according to the manufacturer's instructions (Ambion, Austin, TX). The probes used are as follows: top panel; *hTERT-HA* probe, generated by PCR of the HA tag region of *hTERT-HA* from the pCI-neo-*hTERT-HA* plasmid subcloned into pUHD10-3 (a gift from M Gossen) using the primers 5′-TCCCTCTG-CTACTCCATC-3′ and 5′-GGTTTGTCCAAACTCATC-3′, middle panel; *hTERT* specific probe, generated by PCR amplifying the plasmid PT7T3-*hTERT* containing the 3′ untranslated region of *hTERT* (Meyerson *et al*., 1997) not present in the *hTERT-HA* cDNA, with primers 5′-ACCAGCAGCCCCTGTCACG-3′ and 5′-GGGTGGAAGGCAAAGGAG-3′, third and fourth panels; hTR and actin probes were made as previously described (Meyerson *et al*., 1997). (**b**) Expression of *hTERT-HA* protein in transfected cells. Detergent extracted lysates were prepared as previously described (Kim *et al*., 1994) from positive control 293 cells and two independent GM847 sublines transfected with either pCI-neo or pCI-neo-*hTERT-HA*. 100 μg of each lysate was resolved by SDS–PAGE and immunoblotted with a 1 : 200 dilution of the 12CA5 monoclonal antibody hybridoma supernatant. A ~130 kDa band (arrow, hTERT-HA) was detected in the two independent clones of *hTERT-HA* transfected GM847 cells (lanes 1 and 2) but not in

induced upon telomerase activation which occurs during cellular immortalization (Kilian *et al*., 1997; Meyerson *et al*., 1997) and is down-regulated with telomerase activity during induced differentiation *in vitro* of human HL-60 promyelocytic leukemia cells (Meyerson *et al*., 1997).

Taken together, these data strongly suggest that *hTERT* mRNA expression is a rate-limiting determinant of telomerase enzyme activity in the various cells studied to date. Nonetheless, these results still do not distinguish between two mechanistic hypotheses. *hTERT* gene induction may be the sole rate-limiting determinant of telomerase activity in mammalian cells. Alternatively, induction of *hTERT* expression is only one of a number of events required for telomerase activation. We sought to distinguish between these models by forcing the ectopic expression of *hTERT* mRNA in telomerase-negative cells and gauging the effects of this ectopic expression on the levels of telomerase activity detectable in extracts prepared from these cells.

To do this, we introduced the *hTERT* cDNA into a mammalian expression construct carrying the CMV promoter. In order to distinguish the ectopically expressed hTERT protein from its endogenous counterpart, we marked the C-terminus of the vector-encoded protein with an influenza virus hemagglutinin (HA) epitope tag, yielding the plasmid pCI-neo-*hTERT-HA*. Such a modification does not affect the catalytic activity of the Est2p protein of *S. cerevisiae* (Counter *et al*., 1997).

This *hTERT-HA* construct and a control empty vector were transfected into cells of the SV40-transformed GM847 human fibroblast line (Pereira-Smith and Smith, 1988). In contrast to most immortalized human cells, which appear to activate telomerase in order to maintain telomere length (Shay and Bacchetti, 1997), the immortal GM847 cells are telomerase-negative (Bryan *et al*., 1995). Moreover, while the *hTR* gene is transcribed in these cells (Bryan *et al*., 1997), they lack detectable levels of *hTERT* mRNA (Kilian *et al*., 1997). These observations have led to the conclusion that GM847 cells employ a telomerase-independent mechanism to maintain telomere length (Murnane *et al*., 1994; Bryan *et al*., 1995), perhaps analogous to the recombination-based pathway used by yeast cells to allow chromosomes to maintain telomeres in the absence of a functional telomerase pathway (Lundblad and Blackburn, 1993). Unlike normal, telomerase-negative human somatic cells, which lack replicative immortality, these GM847 cells could be propagated indefinitely following transfection, allowing us to study the properties of clonally isolated cell populations that have stably acquired the introduced *hTERT* gene.

A number of stably transfected GM847 cell clones were generated with either the control vector or the *hTERT-HA* expression vector. mRNA expression of *hTERT* was analysed by RNase protection using probes that specifically recognize either the transfected *hTERT-HA* mRNA or the endogenous *hTERT* transcript. As expected, *hTERT-HA* transcript was detected only in GM847 sublines stably transfected with an *hTERT-HA* expression plasmid (Figure 1a, top panel, lanes 6 and 7), but not in untransfected telomerase-positive control cell lines 293 and HL-60 (lanes 2 and 3), in the parental GM847 line or a GM847 subline transfected with the empty vector (lanes 4 and 5). The cells expressing *hTERT-HA* did not express the endogenous *hTERT* transcript (Figure 1a, second panel, lanes 6 and 7), despite the fact that this RNA is clearly detected in telomerase-positive control cells (lanes 2 and 3). Lastly, in accord with previous observations that the level of the *hTR* RNA subunit of telomerase does not correlate with enzyme activity (Feng *et al*., 1995; Avilion *et al*., 1996), this RNA was detected in all cells tested, irrespective of whether the cells had telomerase activity (Figure 1a, third panel). An actin control probe demonstrates comparable loading of RNA from each cell line (Figure 1a, bottom panel). Moreover, the specificity of the probes used was demonstrated by their failure to protect tRNA (all panels, lane 1).

Expression of hTERT was also analysed at the protein level, by immunoblotting with an anti-HA antibody probe directed against the HA tag of the vector-encoded hTERT protein. A ~130 kDa product corresponding to the predicted size of hTERT was detected in those lines derived from GM847 cells that were stably transfected with the *hTERT-HA* expression construct (Figure 1b, lanes 4 and 5), but not in those cell clones that had been transfected with the empty vector (Figure 1b, lanes 2 and 3). The antibody likewise did not detect endogenous (untagged) *hTERT* (Figure 1b, lane 1) known to be expressed in 293 cells (Meyerson *et al*., 1997), a telomerase-positive control cell line.

We next tested whether these hitherto telomerase-negative GM847 cell lines acquired telomerase activity together with the stable ectopic expression of *hTERT*. Telomerase activity was measured in these different cell lines by assaying the ability of a cellular extract to elongate a primer in a telomerase-specific manner. The products of this *in vitro* reaction are subsequently detected by specific PCR amplification, yielding a ladder of products differing from one another by 6 bp (Kim *et al*., 1994). As shown in Figure 1c, telomerase activity was detected in 293 cells (lanes 3–5), a cell line with one of the highest levels of telomerase activity known. This

two vector-transfected clones (lanes 3 and 4) or 293 cells (lane 5), (**c**) Telomerase activity of GM847 cells expressing *hTERT-HA*. Detergent extracted lysates from 293, parental GM847 cells and GM847 cells transfected with either pCI-neo or pCI-neo-*hTERT-HA* were assayed for telomerase activity by the telomere repeat amplification protocol (Kim *et al*., 1994), according to the manufacturer's instructions (Oncor, Gaithersburg, MD). Telomerase activity was assayed from 0.2 μg of extract isolated from the parental GM847 cell line (lane 7) and two clones of pCI-neo vector-transfected GM847 cells (lanes 9 and 11), or from 0.2, 0.02 and 0.002 μg of extract isolated from positive control 293 cells (lanes 3–5) and two separate pCI-neo-*hTERT-HA* transfected GM847 cell lines (lanes 13–15 and 17–19). As negative controls, lysis buffer alone was assayed for enzymatic activity (lane 1) and 0.2 μg of protein extract derived from all samples was heat treated at 85°C for 5 min (which inactivates telomerase) prior to assaying for telomerase activity (lanes 2, 6, 8, 10, 12, 16). All extract dilutions were done in 1 × CHAPS lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS and 10% glycerol)

activity was sensitive to heat treatment of the extract (lane 2), which inactivates telomerase (Kim *et al*., 1994). In contrast, almost no telomerase products were detectable following assay of extracts from untransfected GM847 cells (lanes 6 and 7) or from GM847 sublines stably transfected with the empty control vector (lanes 8–11). This inability to detect telomerase was not due to the presence of a PCR-inhibiting activity, as an internal control was specifically PCR-amplified (lanes 6–11) (Kim and Wu, 1997).

In marked contrast, telomerase activity was readily detectable in those clones of GM847 cells that were stably transfected with the *hTERT-HA* expression vector (lanes 13–15, 17–19) and this activity was sensitive to heat inactivation (lanes 12 and 16). The levels of telomerase activity observed in these transfectants approached those seen in extracts from 293 cells (lanes 2–5). The restoration of telomerase activity in the cells transfected with *hTERT-HA* was not due to the up-regulation of the endogenous *hTERT* gene as we failed to detect the corresponding mRNA in these cells (Figure 1a, second panel, lanes 6 and 7). Thus, ectopic expression of *hTERT* in previously telomerase-negative cells is sufficient to generate telomerase activity at levels comparable to those found in immortalized telomerase-positive cells.

The telomerase activity detected in cells transfected with the *hTERT-HA* expression vector was physically associated with ectopically produced hTERT-HA, confirming that hTERT is, as predicted, a constituent of the telomerase holoenzyme. As shown in Figure 2, telomerase activity could be immunoprecipitated with an anti-HA monoclonal antibody from extracts of either 293 cells or GM847 cells that ectopically express *hTERT-HA* (Figure 2, lanes 1 and 5). The telomerase activity was not immunoprecipitated with an antibody directed against an irrelevant antigen (anti-p53: Figure 2, lanes 2 and 6), nor was it immunoprecipitated when

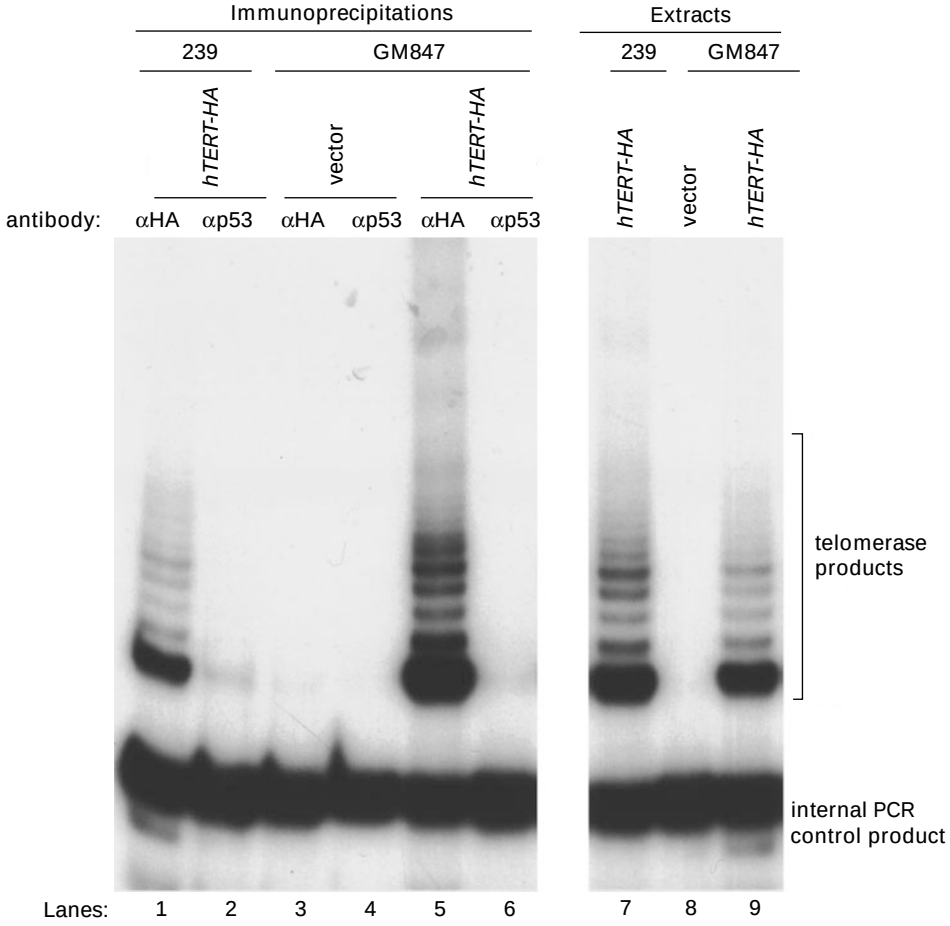

Figure 2 hTERT is a physical constituent of the telomerase holoenzyme. Extracts of *hTERT-HA* transfected 293 cells (lanes 1, 2, 7), vector-transfected GM847 cells (lanes 3, 4, 8), and *hTERT-HA*-transfected GM847 cells (lanes 5, 6, 9), were immunoprecipitated with anti-HA (12CA5) antibodies (lanes 1, 3, 5) or anti-p53 (Pab421) control antibodies (lanes 2, 4, 6), or left untreated (lanes 7, 8, 9) and assayed for telomerase activity. Specifically, 1 μg of cell extract diluted in 100 μl of CHAPS lysis buffer containing 150 mM NaCl was incubated for 1 h at 4°C with 100 μl of hybridoma supernatant, and then precipitated by incubation for 45 min with 50 μl of 20% protein A agarose bead suspension. Immunoprecipitates were washed three times with CHAPS/NaCl buffer, and resuspended in 20 μl of CHAPS/NaCl buffer. 2 μl were assayed for telomerase activity. The *hTERT-HA* transfected 293 cells used in this experiment were derived from transfecting 293 cells with pCI-neo-*hTERT-HA* using the Superfect reagent (Qiagen, Chatsworth, CA), and isolating stable transfected clones on 500 μg/ml of G418

either antibody was incubated with extracts prepared from control vector-transfected GM847 cells (Figure 2, lanes 3 and 4). Taken together, these data indicate that telomerase activity is specifically co-immunoprecipitated with hTERT-HA.

We wished to determine whether the observed induction of telomerase activity following ectopic hTERT expression was unique to GM847 cells. For this reason, we transiently transfected WI-38 normal human fibroblasts, which lack detectable levels of telomerase activity and *hTERT* message but express the hTR gene (Meyerson *et al*., 1997 and not shown), with the *hTERT-HA* expression construct. Since transient transfection of normal human cells is extremely inefficient, we co-transfected the plasmid pGreenLantern-1, which encodes the green fluorescent protein (GFP), together with either the *hTERT-HA* expression vector or the control vector. Cells expressing GFP, and hence quite likely the co-transfected plasmid, were sorted by virtue of their fluorescence.

Extracts derived from both populations of fluorescing cells were assayed for telomerase activity. Whereas fibroblasts transfected with pGreenLantern-1 and pCI-neo vector alone lacked enzymatic activity (Figure 3, lanes 1–4), those co-transfected with the *hTERT-HA* expression vector were clearly telomerase-positive (Figure 3, lanes 5–8). Transfection with pCI-neo-*hTERT-HA* of IMR-90 cells, another telomerase-negative normal human fibroblast cell strain that does not normally express *hTERT* (Kim *et al*., 1994; Meyerson *et al*., 1997), also gave rise to telomerase activity (not shown). Thus, ectopic expression of *hTERT* in these two types of normal human somatic cells results in readily detectable telomerase activity.

Taken together, these data demonstrate that the ectopic expression of hTERT in otherwise telomerase-negative human cells is both necessary and sufficient for induction of telomerase activity. Furthermore, the physical association of hTERT with telomerase activity confirms that hTERT is a telomerase subunit. The fact that forced expression of hTERT sufficed to impart telomerase activity indicates that levels of *hTR* mRNA and TP-1 or other still-unidentified components of the telomerase holoenzyme are not rate-limiting determinants of telomerase activity in these cells. Up-regulation of the *hTERT* gene is therefore the sole barrier to activation of telomerase in the tested cells.

It still remains to be determined if telomerase activity can be restored in this fashion in all telomerase-negative cells. However, we show that activity is conferred by ectopic expression of hTERT in cell types representative of two known classes of telomerase-negative cells: telomerase-negative immortal cell lines and normal mortal human cell strains. Thus, it is likely that expression of *hTERT* mRNA is the rate-determining step for telomerase activation in other human cells lacking enzyme activity. This makes the regulation of transcription from the *hTERT* promoter a potential target for modulation during tumorigenesis and cell immortalization.

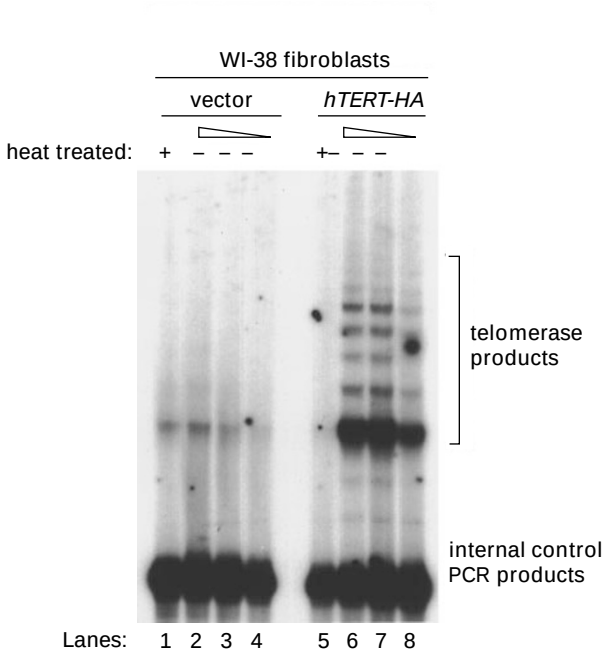

Figure 3 Induction of telomerase activity by transient expression of *hTERT* in WI-38 cells. WI-38 cells were transiently transfected with the plasmid pGreenLantern-1 (Gibco/BRL, Gaithersburg, MD) expressing the green fluorescent protein (GFP) together with either pCI-neo or pCI-neo-*EST2-HA* using the Superfect reagent. Detergent extracted lysates were prepared from the flow-sorted cells exhibiting strong fluorescence at 530 ± 30 nm, which should represent the transfected cells. 2 μl of lysates, diluted to $\sim$1000, 500 and 250 cell equivalents, derived from the flow sorted WI-38 cells co-transfected with pGreenLantern-1 and either the empty vector (lanes 2–4) or pCI-neo-*hTERT-HA* (lanes 6–8) were assayed for telomerase activity. As a negative control, 2 μl of lysate representing $\sim$2000 cells derived from both cell transfections were heat treated at 85°C for 5 min prior to assaying for telomerase activity (lanes 1 and 5)

Acknowledgements
We thank Glenn Paradis, Michael Gennings, and Dr Ante S Lundberg for advice and technical assistance in flow sorting cells, Dr Brian Elenbaas for the 12CA5 and Pab421 antibodies, Dr Olivia Pereira-Smith for the cell line GM847 and Drs Philipp Steiner, Roderick Beijersbergen, William Hahn, Scott Dessain, and the members of the Weinberg laboratory for help, advice, and encouragement. This work was supported by grants from the National Cancer Institute of the USA, OIG CA 39826. Christopher M Counter is supported by an MIT-Merck postdoctoral fellowship and Matthew Meyerson by a Damon Runyon-Walter Winchell Cancer Research Foundation postdoctoral fellowship. Robert A Weinberg is an American Cancer Society Research Professor and a Daniel K Ludwig Cancer Research Professor.

Note added in proof
Since this manuscript was submitted, activation of telomerase by ectopic expression of *hTERT* has also been reported by Weinrich SL *et al*., *Nature Genetics*, **17,** 498–502 (1997) and by Nakayama J *et al*., *Nature Genetics*, **18,** 65–68 (1998). Immunoprecipitation of telomerase activity with hTERT has also been reported by Harrington L *et al*., *Genes & Development*, **11,** 3109–3115 (1997).

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