The hTERT Gene Is Embedded in a Nuclease-resistant Chromatin Domain*

Received for publication, October 5, 2004, and in revised form, October 22, 2004 Published, JBC Papers in Press, October 29, 2004, DOI 10.1074/jbc.M411352200

Shuwen Wang and Jiyue Zhu‡

From the Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Normal human cells rarely undergo spontaneous immortalization. Given that ectopic expression of the human telomerase catalytic subunit hTERT leads to cellular immortalization, the endogenous hTERT gene is likely constitutively repressed. Hence, we have examined the chromatin structure of the native hTERT locus and the neighboring loci, CRR9 and Xtrp2, in normal human fibroblasts and a set of immortal lines. Using generalized DNase I sensitivity assays, we revealed that the entire hTERT gene was embedded in a chromatin domain that was as resistant to the nuclease as the well studied β-globin loci in both telomerase-positive and -negative cells. This condensed domain was at least 10 kb in size and contained the intergenic region 5′ to the hTERT gene and the downstream Xtrp2 locus. A transition from the nuclease-sensitive CRR9 locus to the condensed region appeared near the 3′-end of the CRR9 gene. hTERT transcription was associated with the appearance of a major DNase I-hypersensitive site positioned around the hTERT transcription start site and several minor hypersensitive sites. In telomerase-negative cells, the inhibition of histone deacetylases by trichostatin A led to the opening of this chromatin domain, accompanied by transcription from the hTERT gene but not the Xtrp2 gene. In contrast, the inhibition of protein synthesis by cycloheximide induced transcription from both the hTERT and Xtrp2 genes, indicating that histone deacetylases and labile factors coordinate to silence this chromosomal region. Taken together, our data suggest a novel mechanism of hTERT regulation at the chromatin level and have important implications for studying telomerase expression.

Maintaining the integrity of telomeres is essential for long term cellular proliferation and survival (1). Telomeres are specialized nucleoprotein complexes containing TTAGGG repeats and serve as protective caps of linear chromosomal ends. Telomeres shorten as cells proliferate and are replenished by telomerase, which consists of a catalytic protein subunit, TERT, and an RNA template subunit, TER (2). In most normal human cells, telomerase is either undetectable or expressed at an extremely low level (3). As a result of telomere attrition, these cells will undergo a permanent cell cycle arrest (known as M1 senescence) at the end of their life span (Hayflick limit) (4). When this telomere-dependent cell cycle block is temporarily bypassed by the expression of viral oncoprogens, such as SV40 T antigens, cells continue to lose telomeres and culminate in an M2 crisis in which severe chromosomal instability causes massive cell death (5, 6). Spontaneous immortalization arising from both M1 and M2 proliferative blocks is extremely rare (7, 8). The very few cells (10−7 to 10−8) that survive M2 crisis have attained the ability to maintain their telomeres, either by increasing telomerase or by acquiring a telomerase-independent alternative telomere-lengthening mechanism (ALT) (9). The fact that elevated levels of telomerase activity have been found in vast majority of cancer cells and immortal cell lines (10, 11) suggests that the activation of telomerase is a critical step in tumorigenesis.

Human telomerase activity is regulated at multiple levels, with a major regulatory step at hTERT transcription (12). Both positive and negative regulatory mechanisms of hTERT transcription have been reported. hTERT transcription can be activated by overexpressing several transcription factors such as c-Myc, Sp1, an Ets family protein ER81, and estrogen receptors (13–18). An examination of hTERT promoter sequences revealed the presence of consensus binding elements for these transcription factors, which activated the hTERT promoter in transient reporter assays. In addition, several tumor suppressor pathways have been implicated in the negative regulation of hTERT transcription. For example, E2F-1, Mad1, TGF-β, and Menin regulated the activity of the hTERT promoter and the level of hTERT mRNA (19, 20).

Although results from experiments studying reporter plasmids containing the hTERT promoter have provided insightful information on the transcriptional regulation of hTERT gene, these studies also indicated that the transiently transfected hTERT promoter was not an adequate model for the endogenous hTERT promoter. For example, we have demonstrated previously that transiently transfected plasmid reporters containing −7.4 to +6.5 kb of the hTERT promoter sequence did not correlate with the status of the endogenous hTERT promoter in telomerase-positive and -negative fibroblasts (21). Similarly, Ducrest et al. reported that transiently transfected green fluorescence protein reporters controlled by hTERT promoter sequences were equally active in telomerase-positive and -negative human 21NT breast carcinoma cells (22). Such discrepancies between the activities of transscriptase; hTERT, human TERT; ALT, alternative lengthening of telomere; CRR9, cisplatin-resistance related gene; HS1, DNase I-hypersensitive site; TRAP, telomeric repeat amplification protocol; TSA, trichostatin A; RT, reverse transcription; Xtrp2, a gene encoding an orphan transporter protein.

* This work was supported in part by grants from the W. W. Smith Charitable Trust and the Penn State Cancer Research Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health Grant R01GM071725, recipient of an Research Scholar Award from American Cancer Society, and to whom correspondence should be addressed: Dept. of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, 500 University Dr., Hershey, PA 17033. Tel.: 717-531-3597; Fax: 717-531-7667; E-mail:joz1@psu.edu.

1 The abbreviations used are: TERT, telomerase reverse transcriptase; hTERT, human TERT; ALT, alternative lengthening of telomere; CRR9, cisplatin-resistance related gene; HS1, DNase I-hypersensitive site; TRAP, telomeric repeat amplification protocol; TSA, trichostatin A; RT, reverse transcription; Xtrp2, a gene encoding an orphan transporter protein.

This paper is available on line at http://www.jbc.org
transiently transfected promoters and transcription of the native hTERT promoter are likely resulted from missing distal regulatory elements and/or the lack of native chromatin configuration in the transiently transfected reporters (23). Therefore, understanding the transcriptional regulation of the native hTERT gene is crucial for dissecting the molecular mechanisms of cellular immortalization.

The endogenous hTERT gene is likely repressed in normal human cells. Several previous reports have noted that inhibition of histone deacetylases led to hTERT transcription in telomerase-negative cells (24, 25). However, the molecular mechanisms of hTERT induction by histone deacetylase inhibitors remain elusive. We have recently shown that inhibition of histone deacetylases induced hTERT transcription in the presence of the protein synthesis inhibitor cycloheximide (21). This induction of hTERT transcription was accompanied by the appearance of a major DNase I-hypersensitive site near the transcription start site of the endogenous hTERT promoter. The transcription from a promoter is also determined not only by proximal regulatory elements but also by distal elements and its surrounding chromatin configuration. The human α- and β-globin multi-gene complexes are among the best studied chromosomal regions (26). The α-globin gene cluster is located in a region near the telomere of chromosome 16p, which contains many constitutively expressed genes and is accessible to nucleases in many cell types (27, 28). In contrast, the region containing the β-globin gene cluster on chromosome 11p is sensitive to DNase I only in erythroid cells in which β-globin genes are expressed. This region is in a close conformation and is less accessible to the nucleases in other cell types (29, 30).

To further understand the hTERT regulation and its activation during cellular immortalization, it is necessary to study the endogenous hTERT gene in its native chromatin environment. Utilizing DNase I sensitivity assays, we have mapped the structure of the native hTERT promoter and its chromatin environment in pre-crisis and immortal human fibroblast cells as well as several commonly used cancer cell lines. Our data provides the first detailed mapping of the chromatin environment of the native hTERT gene.

**DNase I Sensitivity of the hTERT Locus**

**Table I**

| Probes | 5′-Primers (5′ → 3′) | 3′-Primers (5′ → 3′) |
|--------|---------------------|---------------------|
| A      | GTTTTTCCATGGGGCGTATG | TTTGCTTTATCCCTGCGTCT |
| B      | GTTTTTCCATGGGGCGTATG | TTTGCTTTATCCCTGCGTCT |
| E      | GGATGGCTTGGCTGCGTCT  | GGATGGCTTGGCTGCGTCT |
| F      | TTGCTGAGTTGAAGGAGGA   | TTGCTGAGTTGAAGGAGGA |
| Gα     | CTTTGCTGCAGTCCCTGTCC | CTTTGCTGCAGTCCCTGTCC |
| H      | CTTTGCTGCAGTCCCTGTCC | CTTTGCTGCAGTCCCTGTCC |
| I      | CACAGATCGCTGCCACATGT  | CACAGATCGCTGCCACATGT |
| J      | CGGTGCAGTCCCTGTCC    | CGGTGCAGTCCCTGTCC |
| K      | CGGTGCAGTCCCTGTCC    | CGGTGCAGTCCCTGTCC |
| L      | GGTGCTTCCACTAGCTCCAG | GGTGCTTCCACTAGCTCCAG |
| M      | TGTGGACGTAGGGGATGAGGA | TGTGGACGTAGGGGATGAGGA |
| N      | TGACCTAGGGGAGTGGAGGA  | TGACCTAGGGGAGTGGAGGA |
| O      | ATGGCTTGTCAGTCCCTGTCC | ATGGCTTGTCAGTCCCTGTCC |
| P      | CAAAAACCTGGCAGTCCTGC  | CAAAAACCTGGCAGTCCTGC |
| Q      | CAAAAACCTGGCAGTCCTGC  | CAAAAACCTGGCAGTCCTGC |
| β-globin | GGTGCTTCCACTAGCTCCAG | GGTGCTTCCACTAGCTCCAG |
| Xtrp2a | TGACTTCTTCCAGGAGTCCG  | TGACTTCTTCCAGGAGTCCG |
| Xtrp2b | TGGTTTTCATGGGGCTGTAG  | TGGTTTTCATGGGGCTGTAG |

* 334-bp XbaI fragment from the PCR product.
* 557-bp DraI fragment.
* 431-bp DraI fragment.

**Materials and Methods**

**Cell Lines**—3A96 (ALT), 3C104a (telomerase-positive), 3C166a (ALT), 3C167b (telomerase-positive), and 3A96 (ALT) immortal lines and pre-crisis cells are derived from IMR90 cells and have been described previously (5, 21). IMR90, HeLa, and 293 cells were obtained described previously (21). IMR90, HeLa, and 293 cells were obtained from the American Type Culture Collection. Pre-crisis cells were infected with the recombinant retrovirus LNCX-c-Myc (31), and neomycin-resistant colonies were pooled to generate 3C/Myc cells. IMR90 and its derived cell lines were cultured in minimal essential medium (Medium) containing 10% fetal bovine serum (Hyclone). HeLa and 293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% newborn calf serum (Mediatech). Clonal pre-crisis cells were generated by transforming IMR90 cells with a plasmid containing SV40 large and small t antigens. Pre-crisis cells underwent M2 crisis upon serial passaging. Immortal cell lines listed on the right arose spontaneously during crisis. The captions telo+ and telo– indicate that cells express detectable and undetectable levels of telomerase, respectively. 3C/Myc was derived from a neomycin-resistant pool of pre-crisis cells infected by the recombinant retrovirus LNCX-c-Myc.

**DNase I Assays**—Isolation of nuclei, DNase I treatment, genomic DNA isolation, and Southern analysis were performed as reported previously (21). Probes were amplified from genomic DNA by PCR (genomic fragments A, B, and E–Q; Table I). For genomic fragments C and D, the 270-bp StuI fragment of the human CRR9 cDNA was used as a probe. Full-length genomic bands were quantified by phoshoflorescence analysis. Generalized DNase I sensitivity was calculated by the equation S = log(Xd/Cd)/log(Xu/Cu) × T, where X and C are test and control band intensities for the undigested (x) or digested (d) samples and T is the size ratio of the control to test fragments (32). Data from one experiment are presented. All of the experiments were repeated, and similar results were obtained.

**RT-PCR**—RNA isolation, cDNA synthesis, and PCR analysis were described in detail previously (21). Primers for hTERT and CRR9 mRNA were published previously (21), and primers for Xtrp2 mRNA are shown in Table I. For hTERT and CRR9 duplex RT-PCR, cDNAs were amplified for 32 cycles, whereas the Xtrp2 cDNA was amplified for 35 cycles.

**Telomerase Assay**—A modified telomeric repeat amplification protocol (TRAP assay) (33) was used to measure telomerase activity, as described previously (21).
RESULTS

Transcription of the hTERT Gene Is Accompanied by the Formation of DNase I Hypersensitive Sites at the hTERT Promoter—In our previous report, we identified a major DNase I-hypersensitive site (HS1) at the proximal region of the hTERT promoter in the SV40-transformed, telomerase-positive immortal cell line 3C167b. This HS1 was absent in both telomerase-negative pre-crisis cells and the ALT immortal line 3C166a. To extend this result and determine the role of DNase I-hypersensitive sites in hTERT transcription, we mapped DNase I hypersensitive sites at the hTERT promoter from a variety of cell types, including IMR90 normal human fibroblasts and immortal fibroblast lines derived from IMR90 cells as well as cancer-derived HeLa and 293 cells. The generation of immortal fibroblast lines has been described previously (5, 21) and is illustrated in Fig. 1.

As shown in Fig. 2, normal human fibroblast IMR90 cells contained no DNase I-hypersensitive sites between the EcoRI and SphI sites of the hTERT promoter from −3918 to +1682 bp relative to the hTERT transcription start site. No DNase I-hypersensitive sites were detected in the telomerase-negative 3A96 ALT line either. In contrast, a major DNase I-hypersensitive site was identified at the proximal region of the hTERT promoter in the hTERT-expressing immortal line 3C104a. This hypersensitive site, named HS1, was located at the same position as the major DNase I-hypersensitive site that was detected in another telomerase-expressing immortal 3C167b (see Fig. 4 in Ref. 21). HS1, which was 3.9 kb from the upstream EcoRI site and 1.7 kb from the downstream SphI site, overlapped with the core hTERT promoter (within 100 bp upstream of the transcription start site). In addition to HS1, several minor DNase I-hypersensitive sites were also identified in 3C104a cells. When probe a was used to hybridize the 5′-end of the restriction fragment, three minor DNase I-hypersensitive bands (2.4, 1.6, and 1.1 kb) were detected. These minor bands corresponded to the presence of hypersensitive sites at approximately −1.5 kb, −2.3 kb, and −2.9 kb upstream of the hTERT transcription start site (Fig. 2A). These hypersensitive sites are also observed in hTERT-expressing 3C167b cells but not in telomerase-negative 3C166a ALT cells and pre-crisis 3C cells (21). Therefore, our results indicated that the events leading to telomerase activation and immortalization of these SV40-transformed human fibroblasts were associated with the remodeling of the chromatin configuration at the hTERT promoter. Because the presence of these DNase I-hypersensitive sites correlated with hTERT transcription, these sites were likely induced by the binding of transcriptional regulatory proteins to regions near these sites.

We have further examined DNase I hypersensitivity of the hTERT promoter in several unrelated cancer-derived cell lines, including HeLa cells, a cervical carcinoma cell line, and 293 cells, a transformed embryonic kidney cell line. The major hypersensitive site HS1 and three minor DNase I-hypersensitive sites upstream of the hTERT promoter could be reproducibly identified in HeLa cells, although the HS1 band in this cell line was weaker than in other immortal lines examined. HS1 was also detected in 293 cells when probe b was used. Because of a polymorphic SphI site at approximately −2.6 kb upstream of the hTERT promoter in 293 cells, probe a could not be used to examine the hTERT promoter. In addition, HS1 was de-
detected in the leukemia cell lines HL60 and U937 cells (data not shown). Thus, we concluded that these DNase I-hypersensitive sites were not unique to the immortal fibroblast cell lines and were likely present in a variety of different cell types that express telomerase.

The hTERT Gene Is Located in a Large Repressive Chromatin Region—The chromatin environment in which a gene resides may play a dominant role in its transcriptional regulation. Within the 120-kb genomic region shown in Fig. 3 there are three genes, CRR9, hTERT, and Xtrp2. The upstream CRR9 gene is constitutively expressed in all cells and tissues examined (21, 34). In contrast, the downstream Xtrp2 gene was reported to express exclusively in the kidney (35). Its expression is undetectable in all of the cells that have been examined except in the cells of kidney origin (data not shown). To understand how the native chromatin environment influences the transcription of the hTERT gene, we set out to map the endogenous chromatin conformation using generalized DNase I sensitivity. The experiments were performed similarly to DNase I hypersensitivity assays, except that the rate of disappearance of a full-length genomic fragment was measured as a function of DNase I concentration. To obtain reliable information using this assay, we undertook several considerations, including those suggested by Saitoh et al. (32). First, because the CRR9 gene is expressed at a similar level in all cell types examined (21, 34), chromosomal fragments within the 5'-half of this gene were used as internal controls. Second, because the kinetics of digestion depends on the length of each fragment, the digestion rate was normalized to the sizes of each restriction fragment. To avoid making excessive size adjustment, restriction fragments with similar lengths were compared wherever possible. For example, in Figs. 3 and 5, larger and smaller fragments were normalized to two adjacent fragments at the 5'-end of the CRR9 gene, the fragments A and B, respectively. Third, because the overall digestion rate of a chromosomal fragment is dominated by digestion at DNase I hypersensitivity sites within the fragment, chromosomal fragments containing detectable DNase I hypersensitivity sites were not included. Finally, all data were also compared with the /H9251- and /H9252-globin loci, the two chromosomal regions that have been studied extensively.

We first examined a pair of immortal cell lines, the telomerase-negative 3C166a ALT cells and the hTERT-expressing 3C167b (Fig. 3). As expected, most of the CRR9 gene was relatively sensitive to DNase I digestion, consistent with its
abundant expression in both cell lines (21). The chromatin became less sensitive toward its 3'-end. The intergenic region between the CRR9 and hTERT genes, the entire hTERT gene, and the Xtrp2 gene were considerably more resistant to DNase I digestion. A transition from the sensitive CRR9 gene to the resistant CRR9/hTERT intergenic region appeared near the 3'-end of the CRR9 gene. This pattern of generalized DNase I sensitivity was conserved between telomerase-negative 3C166a ALT cells and telomerase-positive 3C167b cells. To compare this chromosomal region to the hH9251- and hH9252-globin gene clusters, the same blots were also hybridized to DNA probes specific to these two regions. As shown in Fig. 3, the sensitivity of the SacI restriction fragment spanning the hH9251 gene in the hH9251-globin gene cluster was similar to that of the CRR9 gene. The same result was obtained when a probe specific to the intergenic region between the hH9256 and hH9251 genes in the hH9251 cluster was used (data not shown). In contrast, a SphI-EcoRI fragment within the intergenic region between the A hH9253 and hH9254 genes in the hH9252 cluster was resistant to DNase I, similarly to that of the chromatin region containing the hTERT and Xtrp2 genes (Fig. 5). Therefore, using the hH9251- and hH9252-globin gene clusters as references we revealed that, in both 3C166a and 3C167b cells, the chromatin region containing the hTERT gene was in a condensed conformation, whereas its upstream neighbor, the CRR9 locus, was relatively decondensed.

To extend this result, we analyzed another pair of immortal cell lines, 3A96 ALT cells and 3C104a hTERT-expressing cells. In this experiment we used fewer (but representative) chromosomal fragments. As shown in Fig. 4, the generalized DNase I sensitivity of the 120-kb region in 3A96 cells and 3C104a cells was very similar to those of 3C166a and 3C167b cells. Because the immortal fibroblast lines were derived from SV40-transformed IMR90 cells (Fig. 1), we next examined the hTERT-containing chromatin region in their parental cells, the IMR90 normal human fibroblasts. The results, shown in Fig. 5, revealed that this DNase I-resistant domain also existed in IMR90 cells. Thus, the hTERT gene was embedded in a condensed chromosomal region in all of these fibroblast lines, regardless of the status of hTERT expression.

To determine whether the DNase I-resistant chromosomal region identified in these studies was also present in other cell types, we examined HeLa and 293 cells. The closed chromatin region was clearly observed in HeLa cells (Fig. 6). The boundary between the open CRR9 gene and the closed region was also around the 3'-end of the CRR9 gene. In 293 cells the chromatin in the middle of CRR9/hTERT intergenic region was also highly resistant to DNase I digestion (Fig. 6A, fragment G). However, other regions, including the hTERT and Xtrp2 genes, were less resistant to the nuclease when compared with those in fibroblast cells and HeLa cells. Therefore, the condensed chromatin domain containing the hTERT gene was conserved in cell types other than fibroblasts, although the degree of condensation might vary among different cell types.

Overexpression of c-Myc-induced hTERT Transcription and Chromatin Remodeling—It has been reported that c-Myc overexpression was sufficient to induce hTERT transcription (16, 17, 36). Hence, we infected SV40-transformed pre-crisis cells with a recombinant retrovirus LNCX-c-Myc. A pool of neomycin-resistant cells (3C/Myc) was able to proliferate beyond the M2 crisis point (data not shown). These cells expressed hTERT mRNA as determined by RT-PCR and telomerase activity as measured by a telomeric repeat amplification protocol assay (Fig. 7). As shown in Fig. 2B, both the major (HS1) and minor DNase I-hypersensitive sites identified in the hTERT-expressing 3C104a and
3C167b immortal lines were also present in 3C/Myc cells. The overall DNase I digestion pattern of the chromosomal region containing the \textit{hTERT} gene was, however, not affected by the overexpression of c-Myc (Fig. 5). Thus, our data indicated that overexpression of c-Myc was able to induce local remodeling of the \textit{hTERT} promoter and, thereby, the \textit{hTERT} transcription within this condensed chromatin environment.

Relief of Repression Leads to Transcription of the \textit{hTERT} Gene and Its Downstream \textit{Xtrp2} Gene—It has been shown previously that inhibition of histone deacetylases leads to \textit{hTERT} transcription (21, 25). Because generalized DNase I resistance often correlates with the deacetylation of core histones, we have examined chromatin structure of the chromosomal region containing the \textit{hTERT} gene following the inhibition of histone deacetylases by trichostatin A (TSA). Pre-crisis cells were treated with either TSA or cycloheximide or with both TSA and cycloheximide for 24 h. As shown in Fig. 8A, TSA treatment sensitized the entire chromatin region to DNase I digestion, including the \textit{CRR9} gene (fragment A), the \textit{CRR9} h\textit{TERT} intergenic region (fragment G), the \textit{hTERT} gene (fragment K), and the \textit{Xtrp2} gene (fragment P) as well as the \textit{\alpha}- and \textit{\beta}-globin gene clusters. This opening of chromatin did not require \textit{de novo} protein synthesis, as co-treatment of cells with the protein synthesis inhibitor cycloheximide did not block the TSA effect. Thus, deacetylation of histones was required to maintain the condensed chromatin conformation.

To determine the effect of histone deacetylase inhibition on gene expression, the transcription from \textit{CRR9}, \textit{hTERT}, and \textit{Xtrp2} genes was determined by RT-PCR. As demonstrated previously (21), the \textit{CRR9} gene was constitutively expressed at a similar level in all cell types examined, and its transcription was not affected by treatment with either TSA or cycloheximide (Fig. 8B). Hence, for this experiment the level of \textit{CRR9} mRNA was used as an internal control for each cell type. Consistent with our earlier data (21), \textit{hTERT} transcription was induced by TSA and its induction was not blocked by co-treatment with cycloheximide, indicating that the inhibition of histone deacetylase directly induced \textit{hTERT} transcription. In fact, the treatment of pre-crisis cells with both TSA and cycloheximide led to a synergistic induction of \textit{hTERT} mRNA (Fig. 8B). Surprisingly, the treatment of pre-crisis cells with cycloheximide alone also induced \textit{hTERT} expression. In addition, in 3A96 cells, an ALT immortal line, \textit{hTERT} transcription was also induced by either TSA or cycloheximide. Treatment of these cells with both TSA and cycloheximide resulted in a further increase in the \textit{hTERT} mRNA level. Therefore, these
experiments suggested that, in addition to repression by histone deacetylation, the hTERT transcription might also be suppressed by some labile negative regulatory factors. Because both the \textit{Xtrp2} and \textit{hTERT} genes are localized to the same DNase I-resistant chromosomal domain, we subsequently analyzed the transcription of the \textit{Xtrp2} gene by RT-PCR analysis. Because the \textit{Xtrp2} gene is reported to express only in the kidney (35), we examined the \textit{Xtrp2} expression in 293 cells, a cell line derived from human embryonic kidney cells. Using multiple pairs of \textit{Xtrp2}-specific primers, we found that 293 cells expressed \textit{Xtrp2} mRNA (Fig. 8B). All other cell lines, including pre-crisis, 3A96, 3C166a, 3C167b, and HeLa cells, either did not express \textit{Xtrp2} mRNA or expressed it at a very low level (Fig. 8B and data not shown). The expression of the \textit{Xtrp2} gene in 293 cells concurred with our finding by DNase I sensitivity assay that the \textit{Xtrp2} gene was more accessible to DNase I than in other cells (Fig. 6). Surprisingly, although treatment by TSA relaxed the chromosomal region in which \textit{Xtrp2} resides (Fig. 8A), \textit{Xtrp2} transcription was not induced by TSA, as determined by RT-PCR using several primer pairs specific to various regions of the \textit{Xtrp2} mRNA (Fig. 8B and data not shown). In contrast, \textit{Xtrp2} mRNA was induced by cycloheximide in both pre-crisis and 3A96 cells (Fig. 8B). Furthermore, TSA seemed

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Generalized DNase I sensitivity of chromosomal region containing the \textit{hTERT} locus in HeLa and 293 cells.\ A, a graphic representation of generalized DNase I sensitivity. All of the sensitivity values were normalized to that of fragment A (designated as 1.0).\ B, Southern blots showing the digestion of chromosomal fragments.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Activation of \textit{hTERT} transcription and telomerase activity by c-Myc overexpression.\ A, \textit{hTERT} and CRR9 mRNAs detected by a duplex RT-PCR analysis. 3C/Myc and 3C/LNCX cells were pools of pre-crisis cells infected with recombinant retroviruses, LNCX-c-Myc and the LNCX vector, respectively. 3C167b is an \textit{hTERT}-expressing immortal cell line and is included as a positive control. The three \textit{hTERT}-specific bands likely resulted from alternative splicing.\ B, telomerase activity detected by a telomeric repeat amplification protocol (TRAP) assay. 0.05, 0.25, and 1 \textmu g of cell extracts were used in each set of three assays.} 
\end{figure}
to have an inhibitory effect on the basal Xtrp2 expression in both 3C pre-crisis cells and 3A96 cells, as well as on the cycloheximide-induced Xtrp2 expression in 3A96 cells. In this experiment at least two Xtrp2-specific PCR products were detected, likely a result of alternative splicing. Cycloheximide induced predominately the smaller form in pre-crisis and 3A96 cells, whereas 293 cells expressed only the larger form. Together, these results suggested that the Xtrp2 gene is suppressed predominately by labile repressors. The opening of the chromatin in this region by histone acetylation was neither sufficient nor required for the induction of Xtrp2 transcription.

**DISCUSSION**

The regulation of telomerase expression is critical for multiple biological processes, including cellular aging and cancer. Telomerase is widely expressed in early embryos, and its expression decreases during development and becomes undetectable in the majority of human somatic tissues, starting with the neonatal period (37, 38). Telomerase is expressed at a high level in stem cells and is repressed upon differentiation (39–41). Telomerase is expressed in >90% of human cancers (10). In the past few years, telomerase regulation has been subjected to intense investigation, and one consensus from these studies is that the hTERT gene is repressed in a majority of human somatic tissues. However, the mechanism of hTERT repression remains to be elucidated.

Our current study revealed for the first time that the native hTERT gene was located in a large condensed chromosomal region that was resistant to DNase I digestion. The sensitivity of this region to the nuclease was similar to that of the β-globin gene cluster. In most cells that we examined, this chromatin domain began at the 3′-end of the CRR9 gene and extended beyond the Xtrp2 gene. The 5′-end of the Xtrp2 gene is ~1.25 megabase pairs from the telomere of chromosome 5p, according to the May 2004 assembly of the human genome sequence at the University of California at Santa Cruz Genome Browser. It is possible that this entire region is under the influence of the telomere due to the telomere positional effect (42). There are at least 15 genes located between the hTERT locus and the telomere. It would be interesting to determine the expression patterns of these genes. On the other hand, the α-globin gene cluster is located ~150 kb from the telomere of chromosome 16p, an open chromatin region that contains many constitutively expressed genes (27). Thus, the telomere position effect might not necessarily be responsible for the condensed chromatin configuration of the hTERT locus.

Transcriptional activation of the hTERT promoter did not involve global alteration of the chromatin configuration at the hTERT locus. The hTERT locus was condensed in many hTERT-expressing cells, including 3C167b, 3C104a, 3C/Myc, and HeLa cells. Accompanied by hTERT transcription was the appearance of several DNase I-hypersensitive sites, including HS1 and three minor upstream hypersensitive sites. HS1 overlapped with the hTERT core promoter, which contained several GC boxes, the consensus binding sites for Sp1 family transcrip-
tion factors, and two E-boxes, the binding sites for the Myc family proteins and other basic helix-loop-helix proteins. Both GC boxes and E-boxes have been demonstrated to be important for hTERT transcription. The three upstream minor hypersensitive sites (at –1.6 kb, –2.3 kb, and –2.9 kb) did not coincide with any known regulatory elements. The roles of these DNase I-hypersensitive sites in hTERT transcription remain to be determined.

Generalized DNase I sensitivity correlates with the modification of core histones, such as the acetylation of histones H3 and H4 (43–45). In the telomerase-positive and -negative immortal cells, the level of histone acetylation within the hTERT-containing chromosomal regions remains to be determined. Using a genome-wide mapping technique of histone acetylation, the overall level of histone H3 acetylation across the hTERT and Xtrp2 genes was found to be significantly lower than that of the CRR9 gene in both resting and activated T cells.2 These data suggest that this condensed chromatin domain is also present in T lymphocytes. Interestingly, a peak of histone H3 acetylation was found at the 3′-end of the CRR9 gene in both resting and activated T cells. Because histones H3 and H4 near insulator elements are normally hyperacetylated (45), the presence of this peak of H3 acetylation suggests the existence of an insulator element at the end of the CRR9 gene, separating the open chromatin of the constitutive CRR9 gene from the repressive hTERT and Xtrp2 loci. Insulators function to prevent the spreading of repressive chromatin conformation and are typically marked by DNase I-hypersensitive sites (32). Our examination of several chromosomal fragments in this region did not detect any potential hypersensitive sites. However, the presence of abundant repetitive sequences in this region has made the designing of such experiments more difficult. Thus, additional efforts are needed to identify such boundary elements.

The inhibition of histone deacetylases by TSA induced the relaxation of chromatin and hTERT transcription (Fig. 8). Surprisingly, this relief of chromatin repression was unable to produce any detectable repressors coordinated to suppress hTERT transcription, whereas labile repressors dominantly inhibited Xtrp2 expression.

It has been well documented that telomerase repression is a dominant trait. The fusion of normal human cells (mortal) with telomerase-expressing immortal cells formed telomerase-negative immortal hybrids (46), indicating the presence of telomerase repressors in normal human cells. Our studies were consistent with this conclusion and further suggested that repression of the hTERT promoter involved two mechanisms. The first is the generally repressive and condensed chromatin environment of the hTERT promoter, which may involve global histone deacetylation and can be disrupted by TSA treatment (Fig. 8A). The abundant repetitive elements within the 5′-intergenic region might play a role in the establishment and/or maintenance of this repressive environment. Although hTERT transcription in immortal cells does not require the disruption of this global environment (Figs. 3–5), this repressive environment likely contributes to the tight regulation of the endogenous hTERT promoter. The second mechanism of repression may involve the proximal promoter region and be carried out by labile factors. TSA-induced opening of the chromatin domain was able to induce hTERT transcription in telomerase-negative cells. However, the level of TSA-induced hTERT expression was still lower than that present in telomerase-positive immortal cells (Fig. 8B), which was consistent with the presence of promoter-specific repression in telomerase-negative cells. In telomerase-positive immortal cells, it is presumably the loss of these putative repressors, rather than global chromatin de-condensation, that leads to hTERT transcription (22). Our data, however, do not rule out the possibility that this relief of promoter-specific repression may involve local histone hyperacetylation; future studies will be needed to address this subject.

Our current studies have provided plausible explanations for several long-standing enigmas. First, it has been known for decades that the spontaneous immortalization of normal human somatic cells occurs extremely rarely (8, 47). In fact, the spontaneous immortalization of normal human fibroblasts in culture, in the absence of mutagenesis or oncogene transformation, has never been reported. Because the ectopic expression of hTERT is sufficient to induce the immortalization of multiple types of human cells (5, 48–51), the spontaneous activation of hTERT transcription from its endogenous locus must occur extremely rarely, and the native hTERT promoter must be under constitutive repression. Our finding that the endogenous hTERT promoter was embedded in a condensed chromatin domain is consistent with such a deduction. Second, overexpression of c-Myc activated hTERT transcription and led to cellular immortalization (16, 17, 36). However, c-Myc is expressed in many proliferating human cells that do not contain detectable telomerase activity. Our results indicate that a threshold of c-Myc activity may be needed to counter the repression endured by the native hTERT promoter. Third, the hTERT promoter, as large as 14 kb in size, is highly active in transient transfection assays (21, 22). Its activity was comparable with that of the cytomegalovirus and SV40 promoters in both hTERT-expressing and non-expressing cells (21) (data not shown). Despite that finding, the endogenous hTERT mRNA was expressed at a low level even in telomerase-positive immortal cells and cancer cells, which contained no more than a few copies of hTERT mRNA per cell (22). Although other mechanisms such as insufficient transcriptional elongation and RNA processing might contribute to this low expression, the suppression of the native hTERT promoter by its repressive chromatin environment also provides a probable explanation.

In summary, the current mapping of the native hTERT locus has provided a starting point for understanding the regulation of endogenous hTERT transcription and telomerase activity during development and tumorigenesis. Additional experiments will be required to further dissect the structures of this repressive chromatin domain and understand their roles in the transcription of the hTERT gene as well as other genes within this domain.

Acknowledgments—We thank Sergei Grigoriev for the suggestion of using globin genes as references and Laura Carrel for critically reading the manuscript.

REFERENCES

1. Morin, G. B. (1989) Cell 59, 521–529
2. Greider, C. W., and Blackburn, E. H. (1987) Cell 51, 887–898
3. Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A., Weinberg, R. A., Stewart, S. A., and Hahn, W. C. (2003) Cell 114, 241–253
4. Wright, W. E., and Shay, J. W. (1992) Exp. Gerontol. 27, 383–389
5. Zhu, J., Wang, H., Bishop, J. M., and Blackburn, E. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3723–3728

2Keji Zhao, NHLBI, National Institutes of Health, personal communication.
