αCP1 Mediates Stabilization of hTERT mRNA by Autocrine Human Growth Hormone*

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We herein demonstrate that autocrine human growth hormone production in human mammary carcinoma cells results in increased telomerase activity as a result of specific up-regulation of telomerase catalytic subunit (human telomerase reverse transcriptase (hTERT)) mRNA and protein. This increase in hTERT gene expression is not due to increased transcriptional activation of the hTERT promoter but is the result of increased stability of hTERT mRNA exerted by CU-rich cis-regulatory sequences present in the 3′-untranslated region of TERT mRNA. Autocrine human growth hormone up-regulates two poly(C)-binding proteins, αCP1 and αCP2, which bind to these cis-regulatory elements and stabilize hTERT mRNA. We have therefore demonstrated that post-transcriptional modulation of the level of hTERT mRNA is one mechanism for regulation of cellular telomerase activity.

Chromosomal ends consist of small DNA repeats that are not duplicated faithfully during replication resulting in loss of DNA during each replication. To faithfully replicate chromosomal ends, cells use a reverse transcriptase holoenzyme termed the telomerase complex (1). The template human telomerase RNA (hTR)3 and the reverse transcriptase catalytic subunit (hTERT) are considered to be the major components of the human telomerase complex, because the expression of hTERT and hTR alone is able to restore the majority of telomerase activity. The telomerase complex, because the expression of hTERT and hTR alone is able to restore the majority of telomerase activity (2). Telomerase activity is subject to stringent regulation and is regulated by multiple mechanisms such as hTERT mRNA transcription (3), splicing and maturation (4), protein phosphorylation and transport (5), and subcellular localization of each component of the telomerase complex (6). Telomerase activity is minimal in most somatic cells resulting in chromosome shortening after each proliferative cycle eventually resulting in crisis and cell death (7). However, telomerase activity is readily detected in 90% of all tumor cells (8), in all tissues during early development (9), and in tissues with continuous proliferation, such as the hematopoietic system or the epidermis (10, 11).

mRNA turnover is one important mechanism by which gene expression is regulated (13). Specific interactions between sequences within the mRNA (cis-acting regulatory elements) and cellular RNA-binding proteins (trans-acting factors) regulate ribonuclease action and subsequent mRNA decay rates. The majority of these cis-acting elements are present in the 3′-untranslated region (3′-UTR) of the mRNA. cis-Regulatory elements such as AU-rich elements (AU-rich elements), Iron-responsive element (14), and CU-rich elements (15) within the 3′-UTR have been demonstrated to possess a role in mRNA stability. α-Globin mRNA poly(C)-rich segment-binding proteins (αCPs), also referred to as poly(C)-binding proteins, and hnRNP K bind to CU-rich cis-regulatory elements (16). Two of the five members of poly(C)-binding proteins, αCP1 and αCP2, are highly homologous (16). Both αCP1 and αCP2 bind to pyrimidine-rich elements in the 3′-UTR and stabilize a variety of mRNAs including α-globin and erythropoietin (15, 17).

We have previously demonstrated that autocrine production of hGH in immortalized human mammary epithelial cells concomitantly enhances proliferation and offers protection from apoptosis, forming the basis for abnormal mammary acinar morphogenesis, oncogenic transformation, and tumor formation in vivo (18). Furthermore, autocrine production of hGH, in mammary carcinoma cells with epithelial morphology, promotes mesenchymal cellular morphology, increased cell migration, and increased metalloprotease activity with subsequent acquisition of invasive behavior both in vitro and in vivo (19). Given the described oncogenic potential of autocrine hGH and that both the hGH receptor (20) and telomerase (21) are expressed highly in the human mammary stem cell population compared with differentiated mammary epithelial cells, we have examined the potential regulation of telomerase activity by autocrine hGH.

Herein, we demonstrate that autocrine hGH increases telomerase activity. Autocrine hGH increased hTERT mRNA stability by up-regulation of two poly(C)-binding proteins, αCP1 and αCP2, which bind to specific cis-regulatory sequences in the 3′-UTR of hTERT mRNA. Regulation of post-

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transcriptional stability of hTERT mRNA is therefore one mechanism for cellular modulation of telomerase activity.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—The MCF-7 cell line was obtained from ATCC. The MCF-7 cells were stably transfected either with wild type hGH gene (pMT-hGH) (designated MCF-hGH) or with a translation-deficient hGH (designated MCF-MUT) (55). The MCF-7 cells were cultured at 37 °C in 5% CO2 in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Serum is a stimulator of hTERT transcription because of the presence of mitogens such as epidermal growth factor, insulin-like growth factor, and estrogens (24, 28, 29). We have utilized serum deprivation to minimize hTERT transcriptional activation in the experiments reported herein unless indicated otherwise.

**Plasmids**—The full-length and truncated versions of the 3′-UTR of hTERT were generated by PCR amplification from the cDNA generated from MCF-7 cells using the primers: T3.1F, 5′-ATTCCTAGGTGGCAAGCCCGCCACAC-3′; T3.1R, 5′-TTGGCTCTAGAATG-3′; CP1F, ACTGAATTCATGCACGGAAAGGAAG-3′; CP1R, TGTCTCGAGCTAGCTGCACCCCATGC; TP1forward, 5′-TCTAACCTAACTGAGAA-3′; TP1reverse, 5′-AGAAGATAG-3′; CGGCTGTTACATG-3′; Hsp90βforward, 5′-AACCTAGCCC-TTTGTGGGACG-3′; Hsp90βreverse, 5′-GGGCTTGGAGGTTTCAATGATT-3′; P23forward, 5′-GGTACGATCGAAGGGACTATG-3′; P23reverse, 5′-AAATCCAGGTGACAAATATTCTTTA-3′; SSBFoward, 5′-GTAGGACACAATTATTAGAATTTGTTT-3′; SSBrverse, 5′-GTACCTCAAGTTTCTAAGCTCATATG-3′; RPL22forward, 5′-GCAAGTATTCTGGAAGTTCCTTT-3′; RPL22reverse, 5′-GAGTGCGGACAAGGAGGAG-3′; STAforward, 5′-CTACGCAATT-3′; STAreverse, 5′-ACACATACACATGATGTATGCCTTGAC-3′; DCK1forward, 5′-ATCGAGAAGAGAGACATTACC-3′; DCK1reverse, 5′-TTGTATCTACTGCCCCACCTC-3′; NOLA1forward, 5′-ATAGACCCCATATAAGCTGCTG-3′; NOLA1reverse, 5′-GGCAGACCCAAGTGGGCCGCTC-3′; BActinforward, 5′-CGCTCGGGTAGGATCTTCA-3′; BActinreverse, 5′-CGCTCGGGTAGGATCTTCA-3′.

**Northern Blot Analysis**—Total RNA was extracted using TRIzol reagent (Invitrogen) as described by the manufacturer. 30 µg of total RNA were size-separated on a 1.2% denaturing agarose formaldehyde gel and blotted onto Hybond-N+ nylon membrane (Amersham Biosciences). The RT-PCR product of hTERT, β-actin, and luciferase were gel-purified and used as a template for generating the probes using a high prime DNA labeling kit (Roche Applied Science) according to the manufacturer’s protocol. The blots were hybridized in ExpressHyb buffer (Clontech) at 60–64 °C using denatured hTERT or luciferase probe. The membranes were washed at high stringency (0.1 × SSC (0.015 m NaCl and 0.0015 m sodium citrate, pH 7.0) and 0.1% SDS) for 30 min at 65 °C and exposed to x-ray film at −80 °C for 1–7 days. The membranes were stripped in boiling 0.1% SDS and rebotted with the β-actin probe to measure the β-actin mRNA level, which acted as control.

**mRNA Decay Measurements**—To estimate the mRNA decay rates, transcription was inhibited by adding 5 µg/ml actinomycin D in medium (56). RNA was extracted at the indicated times, and the endogenous hTERT and β-actin mRNA levels were analyzed by Northern blotting. The x-ray film was quantified by densitometer, and the ratio of hTERT to β-actin in each sample was calculated and used to determine the relative amount of specific mRNA remaining in each sample. Linear relationships were ensured by densitometric analysis of increasing amounts of RNA subject to Northern blot analysis and extending above the maximum densitometric reading used for data collection.

**Western Blot Analysis and Confocal Laser Scanning Microscopy**—The following primary antibodies were used. Rabbit anti-hTERT, rabbit anti-p-hnRNP K (Ser102), goat anti-hnRNP K, goat anti-αCP1, and goat anti-αCP2 antibody were from Santa Cruz; rabbit anti-GH antibody was kindly provided by Dr. Parlow (National Institutes of Health NIDDK National Hormone and Peptide Program, Torrance, CA). Nuclear extracts or whole cell extracts were prepared, and Western blots were performed as described previously (38). For immunohistochemistry, the cells were transiently transfected with the indicated plasmids for 12 h and serum-starved for 48 h before fixing them for staining and scanning.
**RESULTS**

**Autocrine hGH Up-regulates Telomerase Activity in a JAK2-dependent Manner**—To examine the potential contribution of autocrine hGH to neoplastic progression of the human mammary epithelial cell and to determine the mechanism by which it results in oncogenic transformation, we examined the effect of autocrine hGH on telomerase activity in human mammary carcinoma cells. MCF7 cells were stably transfected with the wild type hGH gene (MCF-hGH) or a translation-deficient hGH gene where the translation initiation is disabled (MCF-MUT) as described previously (22). Telomerase activity was determined using the telomere repeat amplification protocol using the commercial kit from Intergen/Chemicon. In this method, telomerase in the cell extract adds a number of telomeric repeats onto the 3′ end of a biotinylated telomerase substrate oligonucleotide, which is extended and then amplified by PCR. This extension and amplification generates a ladder of products that is observed by agarose gel electrophoresis and ELISA. As observed by both agarose gel electrophoresis and ELISA (Fig. 1, a and b), MCF-hGH cells exhibit increased telomerase activity compared with MCF-MUT cells. The extracts were treated with RNase A to ensure that the PCR product is generated by RNA-dependent telomerase. Exogenously added hGH (50 nM) was without effect on telomerase activity. We also observed autocrine hGH, but not exogenous hGH, regulation of telomerase activity in a nontransformed, but immortalized, human mammary cell line, MCF-10A (data not shown).

The cellular effects of hGH are primarily mediated by stimulation of JAK2 activity (23). The increased telomerase activity observed in MCF-hGH cells as a consequence of autocrine hGH production was completely inhibited by a specific inhibitor of JAK2 (AG490) (Fig. 1c). Thus, autocrine hGH enhancement of human mammary carcinoma cell telomerase activity is JAK2-dependent.

**Both hTERT mRNA and Protein Levels Are Up-regulated by Autocrine hGH**—Telomerase activity is directly correlated to the expression level of hTERT (8). We therefore examined whether autocrine hGH production in mammary carcinoma cells resulted in up-regulation of hTERT mRNA by semi-quantitative RT-PCR and Northern blot analysis (Fig. 2, a and b) and hTERT protein by Western blot analysis (Fig. 2c). Autocrine hGH production in MCF-hGH cells resulted in increased hTERT mRNA and protein compared with MCF-MUT cells (Fig. 2, b and c). To date, regulation of hTERT mRNA has been demonstrated to be predominantly exerted at the transcriptional level (3). We therefore examined whether the autocrine hGH-stimulated increase in hTERT mRNA was due to increased transcriptional activation of the hTERT promoter by use of a reporter construct containing 3.328 kb of the promoter
5' to the hTERT start site (24). Surprisingly, autocrine hGH did not increase reporter activity from the hTERT promoter (Fig. 2d). In contrast both serum (10%) (Fig. 2e) and 17β-estradiol (10 nM) stimulation (data not shown) of MCF-7 cells resulted in activation of the hTERT promoter. The autocrine hGH-stimu-

**FIGURE 1.** Autocrine hGH increases telomerase activity in a JAK2-dependent manner. MCF-MUT (control, where the ATG start site in the hGH gene for translational initiation is disabled) or MCF-hGH (wild type hGH gene) cells were serum-starved for 24 h and were cultured with or without 50 nM hGH and with or without 20 μM AG490 for an additional 24 h. The protein extracts were analyzed by telomere repeat amplification protocol assay for telomerase activity using electrophoresis (a) or ELISA (b and c). Telomerase activity is increased upon expression of hGH (a). MCF-MUT and MCF-hGH cells were grown in serum-free medium supplemented with 20 μM AG490 for 48 h and analyzed for telomerase activity (c).

hGH Specifically Up-regulates hTERT mRNA

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**FIGURE 2.** Autocrine hGH specifically increases hTERT mRNA and protein levels in human mammary carcinoma cells. mRNA expression of telomerase subunits and telomerase-associated proteins in MCF-MUT and MCF-hGH cells serum-deprived for 24 h and cultured with or without 50 nM hGH for an additional 24 h as determined by semi-quantitative RT-PCR (a). Most telomerase subunits and telomerase-associated proteins remain unchanged (hTR, TP1, Hsp90α, Hsp90β, STAU, DKC1, NOLA1, and P23) or decreased (RPL22 and SSB), whereas hTERT mRNA was increased by autocrine hGH. hTERT expression was also determined by Northern blot (b) and Western blot (c) analysis of MCF-MUT and MCF-hGH cells. hTERT promoter activity (3.328 kb) in MCF-MUT and MCF-hGH cells (d). e, 10% serum stimulation of MCF-7 cells resulted in activation of hTERT promoter, whereas hGH did not increase the reporter activity. The asterisk denotes significance.

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Autocrine hGH-stimulated hTERT mRNA Stability Is Due to cis-Acting Sequences in the 3′-UTR Region—
3′-UTRs are most frequently linked with regulation of mRNA stability (30). Given that autocrine hGH
promoted stabilization of hTERT mRNA, we next localized potential 3′-UTR cis-acting sequences respon-
sible for autocrine hGH-dependent hTERT mRNA stabilization. To this end, we generated a series of reporter plasmids in which the full-length (1–554 bp) and truncated (120–554 and 360–554 bp) versions of the 3′-UTR of hTERT mRNA were fused to the 3′ end of the luciferase open reading frame and cloned into the pCDNA3+ expression vector (Fig. 4a). MCF-
MUT and MCF-hGH cells were transiently transfected with the indicated constructs, and the half-
life of luciferase mRNA was estimated by Northern blot analysis after actinomycin D chase experi-
ments. Luciferase mRNA lacking the surrogate 3′-UTR of hTERT exhibited a similar half-life of ~6 h in both MCF-MUT and MCF-hGH
cells (Fig. 4b). In contrast, luciferase mRNA combined with the full-length hTERT 3′-UTR degraded with a half-life of approx-
itimately 5 h in MCF-MUT cells in contrast to MCF-hGH cells, where the half-life was prolonged for >10 h (Fig. 4c). The last 434 bp (nucleotide positions 120–554) of hTERT 3′-UTR also conferred stability to the luciferase transcript in MCF-hGH cells (half-life >10 h) as compared with that of MCF-MUT cells (half-life ~5 h) (Fig. 4d), similar to that observed with the full-
length 3′-UTR. In contrast, the last 194 bp (position 360–554) of hTERT 3′-UTR failed to provide the differential stability to luciferase mRNA between MCF-MUT and MCF-hGH cells with a half-life of 5 h in both cell lines (Fig. 4e). To further verify the differential stability of luciferase mRNA with the surrogate 3′-UTR of hTERT observed between MCF-MUT and MCF-
hGH cells, we measured the luciferase activity of the various constructs in both cell lines under serum deprivation. Both MCF-MUT and MCF-hGH cells were transfected with the indicated constructs for 6 h followed by serum deprivation for 48 h ± 20 μg/ml AG490, and the luciferase activity was deter-
mined. Consistent with that observed by Northern blot analysis after actinomycin D chase, luciferase activity was higher by
~2-fold in MCF-hGH cells compared with MCF-MUT cells when transfected with the luciferase vectors containing either the full-length or the 120–554-bp fragment of hTERT 3′-UTR. Concordantly, there was no difference in luciferase activity between MCF-hGH and MCF-MUT cells when transfected with the control luciferase vector or the luciferase vector con-
taining the 360–554-bp fragment of hTERT 3′-UTR. Further,
the JAK2 inhibitor AG490 prevented the hTERT 3'-UTR mediated up-regulation of luciferase activity observed in MCF-hGH cells with the full-length and the 120–554-bp fragment of the hTERT 3'-UTR (Fig. 4f). We conclude that the cis-acting regulatory elements contained within nucleotide positions 120–360 bp of the 3'-UTR of hTERT are responsible for the autocrine hGH-dependent stabilization of hTERT mRNA.

**cis-Acting Regulatory Elements from the hTERT 3'-UTR Region Are Bound by Multiple RNA-binding Proteins**—We next proceeded to identify the protein(s) binding to the potential cis-acting regulatory region in the 3'-UTR region of hTERT mRNA. We performed REMSA to determine the formation of RNA-protein complexes by use of different regions of the hTERT 3'-UTR as probes (Fig. 5a). Binding reactions were incubated at 22°C for 30 min, after which 2 units of RNase T1 (Roche Applied Science) was added for 10 min, followed by the addition of heparin (final concentration, 5 mg/ml) (Sigma) for 10 min for all of the REMSA experiments. When REMSA was performed with cytoplasmic extract from MCF-hGH cells using the full-length hTERT 3'-UTR, we observed the formation of five complexes (Fig. 5b). The uppermost complex was observed with all hTERT 3'-UTR probes, despite no overlap, and was considered to be nonspecific. The other four bands (designated as RNA-protein binding complexes (RPC) 1–4) were considered specific because they were observed only with the full-length and 120–554-bp hTERT 3'-UTR probes and were absent when the 3'-120-bp (360–554 bp fragment) of hTERT 3'-UTR was used as probe. Also, when the 5'-201-bp (1–201-bp fragment) of hTERT 3'-UTR was used as probe, no shift bands were seen. Thus, the region between nucleotides 201–360 of the hTERT 3'-UTR is a potential cis-regulatory region and is bound by multiple RNA binding proteins. We therefore next performed REMSA with cytoplasmic extracts from MCF-MUT and MCF-hGH cells using the full-length hTERT 3'-UTR as probe to determine whether autocrine hGH would alter the binding of proteins to the hTERT 3'-UTR. All four RPCs were present at higher intensity in MCF-hGH cell extract compared with that of the MCF-MUT cell extract, whereas the nonspecific band exhibited no difference between these two extracts (Fig. 5c,

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**FIGURE 4.** Autocrine hGH stabilization of hTERT mRNA is due to cis-regulatory elements in the 3'-UTR of hTERT mRNA. a, schematic diagram of the luciferase reporter plasmids depicting the different hTERT 3'-UTR regions fused downstream of the luciferase cDNA. Nucleotide positions of the hTERT 3'-UTR are indicated. a–e, MCF-MUT and MCF-hGH cells were transiently transfected with the indicated constructs, and after 6 h each culture was divided to four subcultures. Each subculture was treated with 5 µg/ml actinomycin D for the indicated times. The total RNA was isolated, and the levels of luciferase and β-actin mRNAs were determined by Northern blot. The ratio of luciferase to β-actin in each sample was calculated by densitometric analysis, and the relative amount of mRNA remaining in each sample was determined. The graphs show the means ± S.D. of three independent experiments. f, MCF-MUT and MCF-hGH cells were transiently transfected with the indicated constructs for 6 h followed by serum deprivation for 48 h ± 20 µM AG490, and luciferase activity was determined.

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**FIGURE 5.** REMSA of cytoplasmic extracts from MCF-MUT and MCF-hGH cells with hTERT 3'-UTR probes. The upper panel (a) shows a schematic representation of the luciferase reporter plasmids with the indicated hTERT 3'-UTR regions, and the lower panels (b–f) show the formation of RNA-protein complexes by use of different regions of the hTERT 3'-UTR as probes. The percentage of mRNA remaining at different time points is shown. The data are representative of three independent experiments.
**hTERT mRNA 3′-UTR Stabilization**

**a**

| T7 hTERT 3′UTR | 1 | 2 | 3 | 4 |
|---------------|---|---|---|---|
| FULL LENGTH   | 1-201 | 120-554 | 360-554 |

**b**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| FREE PROBE | RPC1 | RPC2-4 | FREE PROBE |

| CYTOPLASMIC EXTRACT | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---------------------|---|---|---|---|---|---|---|---|---|---|
| + | + | + | + | + | + | + | + | + | + |

**c**

| MCF-MUT CE | Cold Probe 3 | Cold Poly C | Cold Poly U | Cold Poly A |
|------------|--------------|-------------|-------------|-------------|
| + | + | + | + | + |

**FIGURE 5.** Multiple RNA-binding proteins bind the hTERT 3′-UTR cis-acting regulatory elements. a, schematic diagram of the hTERT 3′-UTR probes. b, cytoplasmic extract was isolated from MCF-hGH cells serum deprived for 24 h and REMSA was performed using the hTERT 3′-UTR probes described above in the presence or absence of MCF-hGH cytoplasmic extract. Four RNA-protein complexes were observed and are indicated as RPC1–4. The formation of RPCs was completely abrogated by excess unlabeled probe-3 (lane 4), poly(C) (lanes 5 and 6), poly(U) (lanes 7 and 8), and poly(A) (lanes 9 and 10). *, non-specific band.

**lanes 2 and 3.** A 100 molar excess of unlabeled probe incorporating the nucleotide region between 120 and 554 bp of the hTERT 3′-UTR completely abrogated the RNA-protein complex formation on the full-length hTERT 3′-UTR (Fig. 5c). Thus, autocrine hGH promotes the formation of a complex between cytoplasmic proteins and the 3′-UTR of hTERT.

Having determined that the hTERT 3′-UTR contained cis-regulatory elements, we examined for potential regulatory sequences by searching the UTR data base (31). We observed multiple CU-rich regions within the 201–554-bp fragment of the 3′-UTR (Fig. 6a). These sequences were entirely homologous with the sequences demonstrated to bind poly(C)-binding proteins involved in the regulation of mRNA stability (15). We therefore first determined whether the four RPCs we observed indeed contain poly(C)-binding proteins. We competed the formation of RPCs with an excess of RNA homopolymers. As observed in Fig. 5c (lanes 5 and 6) both low (10 ng/μl) and high (100 ng/μl) concentration of poly(C) completely abrogated the formation of RPC1. The poly(U) homopolymer partially abrogated the formation of RPC1 but did not affect formation of RPCs 2–4. Poly(A) homopolymer had no effect on the formation of RPC1–4. Poly(A) homopolymer had no effect on the four RPCs indicative that proteins binding to the hTERT 3′-UTR are bona fide poly(C)-binding proteins.

**Poly(C)-binding Proteins αCP1 and αCP2 Bind the hTERT 3′-UTR cis-Acting Regulatory Region.—** There exist two identified groups of mammalian poly(C)-binding proteins, the hnRNPs K (66 kDa) and the αCP proteins (39 kDa) (15). To identify which of these poly(C)-binding proteins bind to the 3′-UTR of hTERT mRNA and maintain its stability, we performed an UVXL experiment to assess the molecular masses of proteins binding to hTERT 3′-UTR using the full-length (1–554 bp) 3′-UTR as a probe. Multiple, distinct RNA-protein complexes with approximate molecular masses of 110, 80, and 45 kDa were observed in cytosolic extracts of MCF-hGH cells. The predominant RNA-protein complex had a molecular mass of 45 kDa, which is closer to the molecular mass of αCP proteins, which is 39 kDa (Fig. 6b, lane 2). This indicated that αCPs may be the predominant RNA-binding proteins interacting with the hTERT 3′-UTR. The possibility that αCP proteins may bind to the hTERT 3′-UTR was determined by repetition of the UVXL experiment with recombinant GST-αCP1 and GST-αCP2 proteins. Although both GST-αCP1 and GST-αCP2 bound to the hTERT 3′-UTR (Fig. 6b, lanes 3 and 4), the binding observed with GST-αCP2 was minimal in comparison. We further confirmed the binding of the αCP1 protein to the 3′-UTR of hTERT by REMSA. GST and GST-αCP1 proteins were analyzed with full-length hTERT 3′-UTR as the probe for RNA-protein complex formation. No RNA-protein complex was formed with GST, but GST-αCP1 (Fig. 6c) formed complexes with hTERT 3′-UTR. The formation of this complex was completely abrogated by excess unlabeled poly(C) and repressed partially by poly(U) but not by poly(A) homopolymers (Fig. 6c). Similar data were observed with GST-αCP2 (data not shown). Furthermore, αCP1 and αCP2 antibodies each separately formed supershifted complexes from the hTERT 3′-UTR full-length (1–554 bp 3′-UTR) probe. As observed in Fig. 6d, both αCP1 and αCP2 antibodies produced a prominent supershift; αCP1 antibody significantly reduced RPC2 and 3, whereas αCP2 antibody significantly reduced RPC4. An antibody to hnRNP K was without effect on the protein complexes binding to the hTERT 3′-UTR. Thus, we conclude that αCP1 and αCP2 are hTERT 3′-UTR-binding proteins.
hTERT mRNA 3′-UTR Stabilization

Poly(C)-binding Proteins αCP1 and αCP2 Stabilize hTERT mRNA—αCPs have been demonstrated to regulate mRNA stability by binding to the 3′-UTR of various mRNAs, such as α-globin (15), collagen α1 (32), and erythropoietin (17). We therefore examined the effect of αCP1 and αCP2 on hTERT mRNA stability. We forced the expression αCP1 and αCP2 proteins in MCF-7 cells by transient transfection and assessed the half-life of hTERT mRNA by Northern blot analysis. Forced expression of αCP1 or αCP2 extended the half-life of hTERT mRNA (~9 h) compared with that of vector transfected control (~5 h) after an actinomycin D chase. Thus, both αCP1 and αCP2 separately stabilized hTERT mRNA (Fig. 7, a and b). We have further verified these results by reporter assay using the luciferase-hTERT 3′-UTR chimeric RNA. MCF-7 cells were co-transfected with either control vector or αCP1 or αCP2 expression vectors together with the luciferase reporter containing the surrogate hTERT 3′-UTR. Forced expression of either αCP1 or αCP2 increased luciferase activity 3-fold when compared with the vector control. In contrast, no change in luciferase activity was observed between control or αCP1- or αCP2-expressing cells co-transfected with luciferase cDNA without hTERT 3′-UTR (Fig. 7c). Therefore, αCP1 and αCP2 regulate hTERT mRNA stability through the hTERT 3′-UTR.

Autocrine hGH Regulates the Poly(C)-binding Proteins αCP1 and αCP2—We next examined the regulatory interactions between autocrine hGH and the poly(C)-binding proteins αCP1, αCP2 as well as hnRNP K. The mRNA and protein levels of αCP1, αCP2, and hnRNP K from MCF-MUT and MCF-hGH cells were analyzed by Northern and Western blotting (Fig. 8, a and b). The levels of αCP1 predominantly but also minimally αCP2 mRNA and protein were increased in MCF-hGH cells expressing autocrine hGH compared with MCF-MUT cells (Fig. 8, a and b). However, autocrine hGH exerted no effect on either the mRNA or the protein level of hnRNP K (Fig. 8, a and b). Because Ser302 phosphorylation of hnRNP K has been demonstrated to decrease its RNA binding activity (33), we also examined Ser302 phosphorylation of hnRNP K in MCF-MUT and MCF-hGH cells by use of a Ser302 phosphorylation-specific hnRNP K antibody. Autocrine hGH did not alter the serine phosphorylation of hnRNP K (Fig. 8b). We also verified the protein expression of αCP1 and αCP2 by confocal laser scanning microscopy after transient transfection of MCF-7 cells with an hGH expression plasmid. In cells with expression of hGH, we observed an increased expression of both αCP1 and αCP2 (Fig. 8c). The increased αCP1 expression observed in MCF-hGH cells as a consequence of autocrine hGH production was abrogated by a specific inhibitor of JAK2 (AG490) (Fig. 8d). Thus, autocrine hGH regulates the expression of αCP1 and αCP2.

αCP1 Mediates Autocrine hGH-stimulated hTERT mRNA Stability—To determine whether αCP1 mediated the autocrine hGH-stimulated increase in hTERT mRNA stability, we generated two siRNA constructs to αCP1. Transient transfection of MCF-7 cells with either the scrambled or the αCP1 siRNAs demonstrated that the siRNA construct 5′-TCGACAAGCCTG-GAGGAGATA-3′ abrogated αCP1 mRNA expression in MCF-7 cells by more than 70% (data not shown). Using this construct we subsequently determined whether αCP1 mediates autocrine hGH-stimulated hTERT mRNA stabilization by
depletion of the autocrine hGH-stimulated increase in \( \alpha \)CP1 using semi-quantitative RT-PCR, immunohistochemistry, as well as luciferase assay. MCF-MUT and MCF-hGH cells were transfected with either the scrambled or \( \alpha \)CP1 siRNA construct, and the mRNA levels were analyzed by semi-quantitative RT-PCR, and the protein expression was analyzed by confocal laser scanning microscopic analysis. For confocal microscopy the transfected cells were identified by the positive staining for GFP, which is present in the siRNA vector. Use of the \( \alpha \)CP1 siRNA abrogated the autocrine hGH-stimulated increase in \( \alpha \)CP1 mRNA and protein (Fig. 9, a and b). MCF-7 cells were subsequently co-transfected with either hGH expression plasmid or control vector, with or without \( \alpha \)CP1 siRNA, together with the luciferase reporter containing the surrogate hTERT 3′-UTR. Forced expression of hGH increased luciferase activity 3.5-fold when compared with the vector control. Depletion of endogenous \( \alpha \)CP1 with the \( \alpha \)CP1 siRNA prevented this autocrine hGH-stimulated increase in luciferase reporter activity (Fig. 9c). Therefore, \( \alpha \)CP1 mediates the hGH-stimulated increase in mRNA stability conferred by the 3′-UTR of hTERT.

We conclude that autocrine hGH up-regulation of \( \alpha \)CP1 protein stabilizes hTERT mRNA via its 3′-UTR.

**DISCUSSION**

Herein we have demonstrated another mechanism by which autocrine hGH in human mammary carcinoma cells regulates...
telomerase activity. It is shown that specific growth hormone-releasing hormone (GHRH) antagonists decrease telomerase activity in a glioblastoma cell line (34). It is of interest to note that the GHRH antagonist-mediated decrease in telomerase activity is also associated with down-regulation of hTERT mRNA and without change in the level of hTR or TP1 mRNA as is observed with autocrine hGH in this study. GHRH antagonists have previously been demonstrated to inhibit autocrine GH production in MXT mouse mammary xenografts (35). Autocrine GHRH in the mammary epithelial cell may therefore stimulate telomerase activity by regulation of autocrine hGH production. Although it is apparent that hTERT is hormonally regulated, the hGH-mediated increase in hTERT gene expression is the result of increased stability of hTERT mRNA exerted by CU-rich cis-regulatory sequences present in the 3' UTR of TERT mRNA. We note here that autocrine and not exogenously added hGH regulates telomerase activity. Specific oncogenic actions of autocrine hGH have previously been described, and mechanisms relating to the specific effect of autocrine hGH have been delineated (18, 19).

Telomerase has been reported to possess cellular functions in addition to its role in maintaining the length of the telomere (36). Evidence indicates that abrogation of telomerase activity inhibits cell proliferation via pathways that are not dependent on telomere erosion (37), and it is possible that autocrine hGH regulation of hTERT mRNA stability may participate in these processes. Indeed, autocrine hGH has been demonstrated to promote both proliferation (22) and survival (38) of human mammary carcinoma cells. In this regard it is interesting that uterine endometrium displays altered telomerase activity in a menstrual cycle-dependent manner despite its somatic origin (41). hGH has also been detected in endometrial epithelial cells (39). Thus, it may be possible that autocrine hGH participates in the regulation of telomerase activity required for temporal specific or cyclical proliferative activity. Concordant with this hypothesis, autocrine GH is expressed in the morula and blastocyst before implantation (40), and we have also detected maximal autocrine GH expression in mouse mammary epithelial cells at puberty (42). Furthermore, the expression of the GH receptor is enriched in adult stem cell populations derived from mammary, hematopoietic, and nervous systems (43). Increased expression of autocrine hGH in mammary neoplasia (44) and simply up-regulation of telomerase activity may therefore simply be a recapitulation of the embryonic or stem cell phenotypic effect of autocrine hGH.

The 3'-UTR of hTERT was replete with CU-rich elements that bind with poly(C)-binding proteins (15). Two distinct subsets of poly(C)-binding proteins are found in mammalian cells, hnRNPs K/J and the αCP proteins (α-complex proteins) (45). The precise mechanism by which these proteins bind mRNA and result in its stabilization is not clear, although they are proposed to protect the endoribonuclease-sensitive site from cleavage by the sequence-specific endoribonuclease (46). Alternatively they are proposed to favor the binding of poly(A)-binding proteins to the poly(A) tail of mRNA (47). All poly(C)-binding proteins are characterized by the presence of three KH (hnRNP K homology) domains that bind to CU-rich RNA sequences (16). Use of REMSA herein demonstrated that all four RPCs formed on hTERT 3'-UTR are abolished in the presence of unlabeled poly(C) homopolymers suggesting that all contain poly(C)-binding proteins. Poly(C)-binding proteins may exist as homodimers or oligomerize with other proteins (48), RPCs 1 and 3 contained αCP1, whereas RPC4 contained αCP2. Based on the molecular masses, there must be other proteins present in RPC1. The identity of other proteins in RPC1 and their functional importance for hTERT mRNA stability remain to be determined.

Regulation of mRNA stability by hGH has hereto not been reported. However, the role of mRNA stability in control of gene expression by other hormones has been demonstrated. For example, estrogen increases the stability of vitellogen mRNA by 30-fold (49), and the hGH related hormone, prolactin, stabilizes casein mRNA by 20-fold (50). In contrast, luteinizing hormone decreases the stability of hCGR mRNA (51). Autocrine hGH-stimulated stabilization of hTERT mRNA reported herein is indicative that mRNA stability will be one mechanism by which hGH regulates gene expression. It is therefore likely that other mRNAs utilizing αCP1 and αCP2 for stabilization will also be modulated by autocrine hGH.

Creation of a tumor cell from a normal cell requires both immortalization and oncogenic transformation (52). Forced expression of hTERT in primary human mammary epithelial
cells (HMECs) has been demonstrated to result in their immortalization (53), although the addition of both insulin and epidermal growth factor were required to maintain proliferation. Immortalization is not sufficient to create an oncogenically transformed cell (54). Together with the fact that the oncogenic transformation in immortalized mammary epithelial cells can be achieved by simple forced expression of the hGH gene (18), the autocrine hGH regulation of telomerase activity described herein is therefore an indication that autocrine hGH may constitute a complete oncogene for the HMEC. However, it remains to be determined whether the autocrine hGH-stimulated increase in telomerase activity will be sufficient for immortalization and allow for subsequent oncogenic transformation.

In conclusion, we have demonstrated that autocrine hGH regulates telomerase activity in human mammary carcinoma cells by increasing hTERT mRNA stability, an effect mediated by binding of poly(C)-binding proteins cCP1 and cCP2 to specific cis-regulatory sequences in the 3'-UTR of hTERT mRNA.

REFERENCES

1. Greider, C. W., and Blackburn, E. H. (1987) Cell 51, 887–898.
2. Nakamura, T. M., and Cech, T. R. (1998) Cell 92, 587–590.
3. Wuu, K. J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack A., Lingner, J., and Dalla-Favera, R. (1999) Nat. Genet. 21, 220–224.
4. Kilian, A., Bovtivel, D. B., Abd, H. E., Hime, G. R., Venter, D. J., Keeve, P. K., Duncan, E. L., Reddell, R. R., and Jefferson, R. A. (1999) Hum. Mol. Genet. 6, 2011–2019.
5. Li, H., Zhao, L., Yang, Z., Funder, J. W., and Liu, J. P. (1998) J. Biol. Chem. 273, 33436–33442.
6. Liu, K., Hodes, R. J., and Weng, N. (2001) J. Immunol. 166, 4826–4830.
7. de Lange, T. (1998) Science 279, 334–335.
8. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, S., Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W., and Shay, J. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2722–2727.
9. Mertani, H. C., Zhu, T., Goh, E. L., Lee, K. O., Morel, G., and Lobie, P. E. (2001) J. Biol. Chem. 276, 21464–21475.
10. Schräficke, M., Scarpellini, F., Poverini, R., Alo, P. L., Rossi, G., and Di Tondo, U. (2004) Am. J. Reprod. Immunol. 51, 112–116.
11. Matusin, M. J., Michael, W. M., and Dreyfuss, G. (1992) Proc. Natl. Acad. Sci. U. S. A. 93, 4674–4681.
12. Chen, C. Y., and Shyu, A. B. (1995) Trends Biochem. Sci. 20, 465–470.
13. Newbury, S. F. (2006) Biochem. Soc. Trans. 34, 30–34.
14. Rouault, T., and Klausner, R. (1997) Curr. Top. Cell Regul. 35, 1–19.
15. Weiss, I. M., and Liebhaber, S. A. (1995) Mol. Cell. Biol. 15, 2457–2465.
16. Makeyev, A. V., and Liebhaber, S. A. (2002) RNA 8, 265–278.
17. Czyzyl-Krzeska, M. F., and Bendixen, A. C. (1999) Blood 93, 2111–2120.
18. Zhu, T., Tempel, E. L., Zhang, X., Lee, K. O., Gluckman, P. D., Mertani, H. C., and Lobie, P. E. (2005) Cancer Res. 65, 317–324.
19. Mukhina, S., Mertani, H. C., Guo, K., Lee, K. O., Gluckman, P. D., and Lobie, P. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 15166–15171.
20. Donut, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J., and Wicha, M. S. (2003) Genes Dev. 17, 1253–1270.
21. Sun, W., Kang, K. S., Morita, I., Trosko, J. E., and Chiang, C. C. (1999) Cancer Res. 59, 6118–6123.
22. Kaulsay, K. K., Mertani, H. C., Tornell, J., Morel, G., Lee, K. O., and Lobie, P. E. (1999) Exp. Cell Res. 250, 35–50.
23. Zhu, T., Goh, E. L., Graichen, R., Ling, L., and Lobie, P. E. (2001) Cell Signal. 13, 599–616.
24. Maida, Y., Kyo, S., Kanaya, T., Wang, Z., Yatabe, N., Tanaka, M., Nakamura, M., Ohmichi, M., Gotoh, N., Murakami, S., and Inoue, M. (2002) Oncogene 21, 4071–4079.
25. Bachand, F., and Autexier, C. (1999) J. Biol. Chem. 274, 38027–38031.