The Latency-associated Nuclear Antigen of Kaposi’s Sarcoma-associated Herpesvirus Transactivates the Telomerase Reverse Transcriptase Promoter*

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Telomerase is a multi-subunit ribonucleoprotein holoenzyme that stabilizes telomere length through the addition of new repeat sequence to the ends of chromosomes. Telomerase reverse transcriptase is the subunit of this complex responsible for the enzymatic activity of telomerase. Expression of the reverse transcriptase is regulated at the level of transcription through the action of transcription factors that target its promoter. Most Kaposi’s sarcoma tumor cells are latently infected with the Kaposi’s sarcoma-associated herpesvirus, and the constitutive expression of a viral-encoded latency-associated nuclear antigen has been shown to be important for the maintenance of the viral episome. The proliferative nature of Kaposi’s sarcoma suggests that this antigen may also play a critical role in viral-mediated oncogenesis. In this study telomerase reverse transcriptase promoter elements cloned into a luciferase reporter plasmid were analyzed to determine the ability of the latency-associated nuclear antigen to regulate transcription. The latency-associated nuclear antigen transactivated the full-length promoter in 293T, 293s, and BJAB cell lines. Furthermore, truncation promoter studies implicated sequence from –130 to +5 in viral-mediated activation. This region contains five Sp1 transcription factor-binding sites. Electrophoretic mobility shift assays indicated that the latency-associated nuclear antigen targets and affects the Sp1-DNA complex in the context of BJAB nuclear extracts.

Telomeric DNA, found at the distal ends of chromosomes, consists of a 6-base pair repeat of the sequence TTAGGG (1). Telomeres have an average length of 5–15 kilobases and function to prevent chromosome degradation, end-to-end chromosome fusions, and chromosome loss (2, 3). Additionally, telomeres can induce cellular senescence when they are critically shortened (4, 5). The telomeres of most human somatic cells shorten with each cycle of chromosome replication because DNA polymerase is unable to completely replicate the lagging strand (6). Telomerase is a ribonucleoprotein enzyme complex that functions to stabilize telomere length via the addition of new repeat sequence (2). Telomerase is active in cells with high regenerative capacity such as lymphocytes, hematopoietic progenitor cells, keratinocytes, and uterine endometrial cells (7–9). Moreover, telomerase is typically active in tumor-derived cell lines as well as in malignant tissues (10–12). The activation of telomerase is a common and perhaps necessary step as cells move beyond replicative crisis to immortality associated with cancer cells (11).

Three subunits of telomerase have been identified. Two components are expressed in virtually all human cells with no correlation to telomerase activity. The first is a RNA component, hTR, that provides the template from which additional repeat sequence is constructed (13, 14). The second is an integral protein component, TP1, the human homologue of the Tetrahymena telomerase P80 gene (15, 16). Neither is down-regulated during cellular differentiation when decreases in telomerase activity are observed (15, 17–19). The third component, hTERT,1 is responsible for the enzymatic activity of telomerase (20, 21). In contrast to hTR and TP1, the expression of hTERT correlates with telomerase activity, and hTERT mRNA is down-regulated during cell differentiation and up-regulated during cell immortalization (9, 10, 22, 23). The expression of hTERT is likely regulated through the action of transcription factors targeting the hTERT promoter. An active hTERT promoter is observed in telomerase-positive cell lines and cancer cells, whereas the promoter is repressed in telomerase-negative cells (24, 25).

The products of known oncogenes and tumor suppressors have been shown to activate and repress, respectively, the hTERT promoter. Following the initial identification of Myc as an activator (25–27), further effects were observed in studies with the transactivator Sp1 and with the repressors WT1, Mad1, p53, and MZF-2, all shown to modulate the activity of the hTERT promoter (28–32). Although the E6 gene product of human papillomavirus type 16 has been shown to activate telomerase by functional assays (33), to date no tumor virus gene product has been shown to specifically activate the hTERT promoter.

Prior to cases related to the AIDS epidemic of the early 1980s, KS was a relatively rare skin neoplasm typically afflicting elderly men of Mediterranean descent, immunosuppressed individuals such as transplant recipients, and young men of...
sub-Saharan African countries (34, 35). The high prevalence of KS among HIV-infected homosexual men and the low prevalence among HIV-infected hemophilics and intravenous drug users strongly implicated transmission of an infectious agent through sexual activity (36, 37). KSHV, also referred to as human herpesvirus 8, was subsequently identified by polymerase chain reaction-based studies and designated a γ-herpesvirus placing it in the same family as the primate Rhadinovirus herpesvirus saimiri and the human Lymphocryptovirus Epstein-Barr virus (38, 39). KSHV is strongly linked to Kaposi’s sarcoma with infected individuals converting to seropositivity prior to expressing a disease phenotype (40). There is a greater than 90% correlation between virus nucleic acid detection and disease (41). KSHV also targets the endothelial-derived spindle cell, which is the primary tumor cell associated with KS (42, 43). KSHV has since been associated with other malignancies including multifocal Castleman’s disease and a rare B cell lymphoma, the body cavity-based lymphoma or primary effusion lymphoma (44–46). Body cavity-based lymphoma-derived cell lines are the only KSHV-infected cells easily amenable to study in culture, and KSHV infection in these cell lines is predominantly latent (47). More recently, human-derived microvascular endothelial cells have been shown to support latent infection with KSHV. These cells undergo morphological changes resulting in a phenotype that is similar to that seen in the formation of spindle cells in KS (48). Additionally, KSHV has been shown to infect a variety of cultured cells, including HEK 293, by reverse transcription-polymerase chain reaction detection of a spliced late mRNA (49). KSHV has also been detected by polymerase chain reaction in infected HEK 293 cells but not uninfected ones during serial passage (50).

LANA is a highly immunogenic protein encoded by open reading frame 73 of the KSHV genome (51). The 1162-amino acid LANA protein has definable domains that include an amino-terminal proline-rich region, an 100-amino acid acidic domain, a large glutamine-rich repetitive domain, and a leucine zipper motif (see Fig. 1; Refs. 39 and 52). Additionally, LANA possesses potential nuclear localization signals and numerous phosphorylation sites recognized by several common kinases (53, 54). LANA is constitutively expressed during viral latency and is important for maintenance of the viral episome during chromosome replication (55, 56). The proliferative nature of KS and other KSHV-associated diseases as well as structural motifs of LANA that potentially interact with a variety of cellular factors suggest a role for LANA in mediating viral oncogenesis through transcriptional regulation. Recently, it was shown that LANA antagonizes the tumor suppressor p53, thereby protecting against cell death (57). LANA has also been shown to target the retinoblastoma protein regulating E2F responsive promoters (58). Here, we show that LANA likely contributes to cell immortalization by transactivation of the hTERT promoter.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Lines, and Culture Conditions**—The preparation of pGL3b-hTERT luciferase reporter constructs and the pA3M-LANA expression construct have been described previously (25, 56). The pGL3b-hTERT constructs were obtained from J. Carl Barrett. HEK 293 cells are human embryonic kidney cells transformed by adenovirus type 5 DNA; the HEK 293T cell line is an HEK 293-derived line that stably expresses the SV40 T-antigen (59). BJAB is a B cell derived from a patient with Epstein-Barr virus-negative African Burkitt’s lymphoma (60). BJAB and Rat-1 cell lines were obtained from Elliott Kieff. SUSM-1 cells maintain their telomeres by a telomerase-independent mechanism and are consequently negative for telomerase activity (61).

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The SUSM-1 cell line was prepared by mutagen treatment of fetal human diploid fibroblasts (62). SUSM-1 cells were provided by Masayoshi Namba. BC-3 is a KSHV-positive body cavity-based lymphoma-derived cell line obtained from the American Type Culture Collection (63). HEK 293, HEK 293T, Rat-1, and SUSM-1 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 25 units/ml penicillin, 25 μg/ml streptomycin, and 10 μg/ml gentamicin. BJAB cells were grown in RPMI medium 1640 (Life Technologies, Inc.) supplemented with 10% Dulbecco’s modified Eagle’s medium. BC-3 cells were grown in RPMI medium 1640 supplemented as for Dulbecco’s modified Eagle’s medium but with 20% fetal bovine serum. Cells were grown at 37°C in a humidified environment supplemented with 5% CO2.

**Transfection and Luciferase Assay—**HEK 293, HEK 293T, Rat-1, and SUSM-1 cells were collected at 70% confluency by trypsinization with trypsin-EDTA (Life Technologies, Inc.). 10 million cells were resuspended, along with plasmid DNA (generally 5 μg of pGL3-Basic reporter DNA, 2 μg of pA3M-LANA expression construct), in 1 μl of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were transfected by electroporation with the Bio-Rad Gene Pulser II at 210 V and 975 microfarads. Transfected cells were transferred to 100-mm plates in 10 ml of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The plates were incubated at 37°C in a humidified environment supplemented with 5% CO2 for 20 h. BJAB cells were collected at 5 × 104 cells/ml. 10 million cells were transfected at 220 V and 975 microfarads.

At 20 h, cells were harvested and washed once with phosphate-buffered saline (Life Technologies, Inc.). Luciferase activity was determined as per manufacturer’s instructions using the luciferase assay system with reporter lysis buffer (Promega). Briefly, 200 μl of lysates were prepared by freeze/thaw in reporter lysis buffer. The lysates were subsequently diluted 1:10 in reporter lysis buffer, and 40 μl of the dilution was mixed with 100 μl of luciferase assay reagent. Luminescence was measured for 10 s by the Optiromp I luminometer (MGM Instruments, Inc.). It should be emphasized that lysates were always diluted to ensure that luciferase activity was within the linear range of the assay. The results shown represent experiments performed in triplicate. The experiments were repeated multiple times to ensure that the observed trends were reproducible.

**Western Blot Analysis—**The same lysates prepared for luciferase assays were used and transferred onto nitrocellulose membranes with a semi-dry apparatus. Membrane was blocked with 5% dry milk in PBS and was subsequently blotted with human serum, adsorbed with B cell extracts, and previously shown to react with LANA (66). Western blots were reacted with goat anti-human antibody or FITC-conjugated goat anti-human antibody or FITC-conjugated goat antihuman antibody and washed thoroughly with PBS, slides were incubated with either FITC-conjugated goat anti-human antibody or FITC-conjugated goat antihuman antibody at 1:1000 dilution for 1 h. Slides were washed thoroughly with PBS, and proteins were visualized on an Olympus BX60 fluorescence microscope. The photographs were captured using an Olympus digital camera and the Esprit program version 1.2.

**In Vitro DNA Binding—**Sp1 DNA probes were prepared by annealing complementary oligonucleotides containing the GC-rich Sp1 DNA binding site (Life Technologies, Inc. custom primers). The wild-type probe sequence was taken from –119 to –98 of the hTERT promoter (5′-CCCGACCCCCTTCCCGC-3′). The mutant probe sequence was 5′-CGCCCAGCCGGTCCCGCCG-3′. Probes were end-labeled with [α-32P]dGTP. Sp1 mouse monoclonal IgG at 1:500 dilution for 1 h. Slides were washed thoroughly with PBS, and proteins were visualized on an Olympus BX60 fluorescence microscope. The photographs were captured using an Olympus digital camera and the Esprit program version 1.2.
LANA Transactivates the hTERT Promoter

RESULTS

LANA Activates Transcription of the Full-length hTERT Promoter in HEK 293 Cells—The proliferative nature of cells latently infected with KSHV as well as structural domains of LANA having the potential to mediate protein-protein and protein-DNA interactions suggest a role for LANA in regulating cellular gene expression (Fig. 1). Because expression of hTERT is primarily regulated at the transcriptional level, the effect of LANA on hTERT expression was investigated by testing the ability of LANA to regulate expression from luciferase reporter constructs containing the hTERT promoter in HEK 293 cells. The HEK 293 cell line has been employed in previous studies of KSHV infectivity and propagation. In one study KSHV was cytotoxic to 293 cells and was detected by polymerase chain reaction in infected cells but not uninfected ones during serial passage (50). In another study 293 cells were susceptible to KSHV infection by detection of a spliced late mRNA (49). These data support the use of the HEK 293 cell line for preliminary examination of potential LANA effects on gene expression. The region −1665 to +5 of the hTERT promoter was cloned into the pGL3-Basic luciferase reporter construct such that initiation of transcription via the hTERT promoter would drive the transcription and ultimately the translation of the luciferase gene (25). This reporter, pGL3B-TRTP, was transfected into HEK 293 cells along with pA3M-LANA, a Myc-tagged LANA expression vector. Initial cotransfection of LANA expression vector with the hTERT reporter construct consistently resulted in 4.5-fold activation relative to reporter construct alone. Increasing the concentration of the LANA expression construct consistently augmented the activation to 7-fold. These data indicate that the transactivation of the hTERT promoter in the HEK 293 cell line is directly proportional to the quantity of LANA expressed as demonstrated by the observed dose-response relationship (Fig. 2). Further increasing the amounts of LANA resulted in abrogation of this activity and increased cell death (data not shown).

The hTERT Promoter Element Is Endogenously Activated in Human Cell Lines—To assess the possibility that the above response was due to either the total amount of DNA transfected or the particular cell line employed, similar assays were performed in five cell lines, HEK 293 and 293T, BJAB, Rat-1 fibroblasts, and SUSM-1 fibroblasts. HEK 293, HEK 293T, BJAB, and Rat-1 fibroblasts have significant endogenous telomerase activity. In contrast, SUSM-1 fibroblasts maintain their telomeres by a telomerase-independent mechanism and were previously shown to have little or no telomerase activity (61). In HEK 293T and 293 cell lines, a 15-fold increase in luciferase activity was observed when the telomerase promoter was cloned upstream of the luciferase gene. Similarly, the BJAB cell line showed a 20-fold increase in activation (Fig. 3A). It should be noted that pGL3-Basic background activity differed in these three cell lines and that fold activation of the hTERT promoter was always calculated relative to the appropriate pGL3-Basic/pA3M vector alone control.

To demonstrate that expression of LANA further augments transcription at the hTERT promoter, the aforementioned cell lines were transfected with pGL3B-TRTP luciferase reporter and either LANA expression plasmid or vector control. In HEK 293T and BJAB cell lines, expression of LANA consistently resulted in an additional 1.5–2-fold activation of the hTERT promoter over background activity. Moreover, in HEK 293 cells expression of LANA resulted in 5–7-fold activation relative to background (Fig. 3B). These data indicate that LANA can activate the hTERT promoter over endogenous hTERT promoter activity in these telomerase-positive cell lines and that this effect is not due to the total amount of DNA transfected. As discussed in more detail below, the modest activation observed in HEK 293T and BJAB cell lines was expected because these lines express potential activators of the hTERT promoter, SV40 T-antigen and Myc, respectively. Just as expression of the T-antigen overwhelms LANA-mediated activation of the hTERT promoter in the HEK 293 background, Myc expression likely limits potential fold activation of the hTERT promoter in the BJAB cell line.

In comparison, Rat-1 fibroblasts and the telomerase-negative fibroblast cell line SUSM-1 were examined for LANA-mediated activation of the hTERT promoter. No significant activation over endogenous hTERT promoter activity was observed in either fibroblast cell line (Fig. 3B). It should be noted that although SUSM-1 demonstrated less endogenous hTERT promoter activity than the other cell lines examined (Fig. 3A), in our experiments the activation seen was greater than expected based on previous reports (25, 61). Additionally, it was observed that as the SUSM-1 cell line was expanded in culture for the transfection experiment, the cells acquired a more quickly growing phenotype. This change in phenotype is perhaps attributable to changes in cellular gene expression that...
partially activated the hTERT promoter explaining the significant endogenous activation observed by luciferase assay.

**Transiently Transfected LANA Is Expressed in Human Cell Lines**—To demonstrate that the LANA expression vector employed here, pA3M-LANA, effectively expresses LANA protein in our transient reporter assay, cell lysates from a number of cell lines were examined by immunoblot and immunofluorescence. The results demonstrated a series of bands above the 215-kDa marker in pA3M-LANA-transfected HEK 293T and 293 cells; no bands were present in pA3M control lanes (Fig. 4A, lanes 2 and 4 and lanes 1 and 3, respectively). Protein corresponding to 1.5 million HEK 293 cells was necessary to mimic the LANA intensity of 0.5 million HEK 293T cells, indicating that LANA expression was enhanced in HEK 293T relative to HEK 293, probably because of the presence of the SV40 T-antigen (Fig. 4A, lanes 2 and 4).

To compare the level of expression in transiently transfected cells with KSHV-infected cells, immunofluorescence analysis was performed. Aliquots of BJAB cells were transfected as for hTERT luciferase assays, and slides were prepared for both transfected BJAB and BC-3, a KSHV-positive body cavity-based lymphoma-derived cell line (63). In BC-3 and LANA-transfected cells, FITC-mediated fluorescence was localized to the nucleus (Fig. 4B, compare left and right panels). To demonstrate that this fluorescence was specific for the Myc-tagged LANA expressed here, Myc monoclonal IgG was used for both pA3M and pA3M-LANA-transfected BJAB. Nuclear localization of the fluorescence signal was observed for pA3M-LANA-transfected BJAB but not for the pA3M-negative control (Fig. 4C, compare left and right panels).

**LANA Activates Transcription of Truncated hTERT Promoter Constructs**—Since the recent cloning of the hTERT promoter, the products of known oncogenes and tumor suppressors have been shown to both activate and repress the hTERT promoter via specific protein-DNA interactions (25, 28–32). To examine which region or regions of the hTERT promoter are responsible for LANA-mediated activation, reporter plasmids, with serial truncations of the hTERT promoter cloned upstream of the luciferase gene (25), were cotransfected with LANA expression plasmid in HEK 293 cells (Fig. 5A). In all cases, both endogenous activity and LANA-enhanced activity were normalized as fold activation relative to pGL3-Basic/PA3M vector control as shown in the left column in panels B–F of Fig. 5. The endogenous or LANA-independent activation of these hTERT truncations increased significantly with the removal of 5’ sequence to position −408, perhaps attributable to elimination of repressor binding (Fig. 5, compare B and C, middle columns). Subsequent removal of 5’ sequence to −149 depressed endogenous activation −2-fold (Fig. 5, compare D and E, middle columns). This is consistent with previous reports that the elimination of the region from −208 to −149 and specifically the E-box that it contains depresses endogenous hTERT activation (25). Despite variations in the endogenous activation of the hTERT promoter with serial 5’ deletion, LANA consistently activated transcription relative to background activity in all constructs examined. To demonstrate that LANA does not similarly activate transcription of the luciferase gene in the context of a pGL3-Basic vector with no hTERT sequence in-
serted (Fig. 6). These two observations suggest that the smallest hTERT promoter truncation examined, the −130 to +5 fragment, is sufficient to mediate LANA activation of the hTERT promoter relative to endogenous activation.

LANA Targets the Sp1-DNA Interaction in the Context of the hTERT Promoter—The −130 to +5 region of the hTERT promoter is significant in that it contains five GC-rich boxes (25). Previous electrophoretic mobility shift assay experiments have demonstrated binding of transcription factor Sp1 to these five sites (24); further, it has been suggested that Myc, via the aforementioned E-box, and Sp1 are the major determinants of endogenous hTERT expression (28). To ask the question of whether LANA activates the hTERT promoter via a GC-box-mediated interaction, a probe was designed encompassing a single GC-box. The sequence for the double-stranded DNA probe was taken from −119 to −98 of the hTERT promoter and was chosen because this GC-box matches exactly the consensus Sp1 binding sequence. By electrophoretic mobility shift assay, the probe interacted with BJAB nuclear extracts to yield at least two GC-box-specific bands (Fig. 7, arrows on left). These bands were designated as specific shifts because they both disappeared with GC-box mutant probe (Fig. 7, right panel). Additionally, these two specific complexes were competed by the addition of 200× specific competitor; however, these bands were not competed by nonspecific DNA competitor (Fig. 7, left panel). Furthermore, one of the specific bands was supershifted with Sp1 monoclonal antibody (Fig. 7, asterisk). In the presence of in vitro translated LANA, this Sp1-specific signal was significantly ablated along with the other GC-box-specific bands (Fig. 7, arrows). This ablation was expected because a new LANA complex would likely be too large to migrate significantly on the gel. It should be noted that these GC-box-specific signals were not affected by the addition of nonspecific competitor protein. The small inset at the left of Fig. 7 shows the LANA-specific signal from the in vitro translated LANA used in this assay. Taken as a whole, these data suggest that the
endogenous DNA-protein complex formed at the GC-box and including Sp1 is targeted by LANA.

DISCUSSION

Latent infection with KSHV is believed to play a causal role in several proliferative lesions, namely KS, body cavity-based lymphoma, and multicentric Castleman’s disease (40, 44–46). KSHV also transforms primary human endothelial cells in vitro, and by telomeric repeat amplification protocol assay it was demonstrated that these KSHV-transformed cells had enhanced telomerase activity; telomerase activity was not detected in uninfected cells (43). Although the E6 gene product of human papillomavirus type 16 activates telomerase by telomeric repeat amplification protocol assay (33), the effect of other viruses and their gene products on the regulation of telomerase activity remains relatively unexplored. Here we propose that LANA, a constitutively expressed protein detected in latent KSHV infection, potentially contributes to primary cell transformation and the proliferative nature of KSHV-infected lesions by activating the hTERT promoter.

Just as KSHV does not mediate transformation of all cell types infected, LANA-mediated activation of the hTERT promoter was not equally efficacious in all cell lines examined here. The most dramatic activation was observed in the HEK 293 cell line as LANA activated the wild-type hTERT promoter 5–7-fold. In contrast, activation was 2-fold in HEK 293T and BJAB cell lines. This discrepancy was expected and can likely be attributed to differences in endogenous Myc expression and subsequent activation of the hTERT promoter in these cell lines. BJAB is a B cell line derived from a patient with Epstein-Barr virus-negative African Burkitt’s lymphoma (60). African Burkitt’s lymphoma is notable for translocation of the Myc gene into the immunoglobulin heavy chain locus resulting in constitutive high level Myc expression (67). Myc expression is known to strongly activate the hTERT promoter (25). Consequently, in the BJAB cell line, LANA-mediated fold activation was likely less dramatic because of this already strong endogenous activation mediated by Myc. Similarly, HEK 293T expresses the SV40 T-antigen, a promiscuous transactivator that has been shown to activate the human Myc promoter (68). In fact, endogenous activation of the hTERT promoter was 10-fold higher in HEK 293T relative to HEK 293 by transient transfection luciferase assay, suggesting that endogenous hTERT promoter activity was indeed up-regulated in the HEK 293T
cell line (data not shown). Although the cell lines employed here provide a good initial screen of LANA-mediated hTERT expression effects, we are currently planning further experiments to examine these effects in endothelial cells and primary B cells, cell lines particularly relevant to latent KSHV infection.

Ideally, the cell lines employed in these studies would have undetectable telomerase activity and, consequently, minimal endogenous activation of the hTERT promoter; however, because up-regulation of telomerase activity is a common step in cell immortalization, most cell lines amenable to the assays utilized here have significant detectable telomerase activity. An exception is the SUSM-1 cell line. This line was derived by mutagen treatment of fetal human diploid fibroblasts and maintains telomere length by a telomerase-independent mechanism (61). However, transient transfection experiments performed to assay for LANA-mediated hTERT promoter activation indicated that neither SUSM-1 nor Rat-1 fibroblast cell lines facilitated activation. This suggests that cell-specific factors in the human embryonic kidney and BJAB cell lines necessary for LANA-mediated activation were absent or sequestered in these fibroblast cell lines. As was mentioned previously, all cell lines examined, including the telomerase-negative cell line SUSM-1 (61), showed significant endogenous activation of the hTERT promoter (Fig. 3A). It would be of interest to further examine LANA-mediated activation of the hTERT promoter in a human embryonic or B cell line lacking endogenous hTERT promoter activity.

Promoter truncation studies implicated the −130 to +5 region of the hTERT promoter in LANA-mediated activation. This region was important in that it contained five GC-rich boxes, the consensus binding sites for transcription factor Sp1. Electrophoretic mobility shift assays were performed to assess the interaction of LANA using a probe containing a selected GC-box. The data presented here suggest that LANA alters a specific interaction between Sp1 and the GC-box. However, neither a LANA-specific shift nor a supershifted complex in the presence of LANA was observed. Although Sp1, a 558-amino acid protein, was amenable to gel shift assays resulting in specific shifted complexes and supershifted complexes, LANA, a 1162-amino acid protein, would likely be difficult to assay by this method. One explanation for our data is that LANA interacts with the Sp1-DNA complex, creating a new complex that is too large to migrate significantly on the gel. Alternatively, LANA might be recruited to the GC-box displacing the Sp1 complex, creating new complexes and releasing the bound DNA. It should, however, be noted there was no specific interaction between LANA and the GC-box in the absence of BJAB nuclear extract. Overall, these data suggest that the endogenous DNA-protein complex formed at the GC-box is targeted by LANA, potentially implicating the GC-box in LANA-mediated activation of the hTERT promoter.

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FIG. 7. LANA targets the Sp1-DNA complex. The probe for the left panel consisted of an Sp1 consensus sequence (GC-box) and flanking sequence taken from −119 to −8 of the hTERT promoter; this probe represents one of five Sp1 binding sites from −130 to +5 of the hTERT promoter. First and second lanes, probe with or without BJAB nuclear extract; third and fourth lanes, probe with nuclear extract and Sp1 mouse monoclonal IgG as indicated; fifth and sixth lanes, probe with nuclear extract and either unprogrammed rabbit reticulocyte lysate or in vitro translated LANA; seventh and eighth lanes, probe with nuclear extract and 200-fold molar excess either specific or irrelevant cold probe. The probe for the right panel differed from the wild-type probe used in the left panel by a CC to AA mutation in the Sp1 consensus sequence. The additions to the right panel were as described for the left panel. The inset at the lower left shows in vitro translation of pA3M-LANA (lane 2) in the presence of 35S-labeled Cys and Met versus negative control (lane 1). The arrows on the left indicate the positions of the GC-box-specific shifts. The asterisk indicates the position of the supershifted complex in the presence of the Sp1 antibody.