A Transcriptional Inhibitor Induced in Human Melanoma Cells upon Ultraviolet Irradiation

Yang M. Yang, Susan E. Rutberg, Fen-Chi Luo, Thomas E. Spratt, Ruth Halaban, Soldano Ferrone, and Zeev Ronai*

Molecular Carcinogenesis Program [Y. M. Y., S. E. R., F-C. L., Z. R.] and Division of Chemical Carcinogenesis [T. E. S.], American Health Foundation, Valhalla, New York 10595; Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut 06510 [R. H.]; and Department of Microbiology and Immunology, New York Medical College, Valhalla, New York 10595 [S. F.]

Abstract

Nuclear proteins from human melanoma cells exhibit strong binding activity to the UV response element (TGACAACA); however, this binding is inhibited following UV-C irradiation. In contrast, the binding of nuclear proteins from rodent fibroblasts and human keratinocytes to the UV-responsive element is initially weak and increases significantly upon UV irradiation. The addition of nuclear proteins from UV-irradiated melanoma cells to those prepared from nonirradiated cells inhibited the binding to the UV-responsive element in a concentration-dependent manner. Fast protein liquid chromatography analysis of nuclear proteins from UV-irradiated melanoma cells revealed 12 and 14 kilodalton proteins within a fraction which also contained the inhibitory activity. The inhibitor blocks the binding of proteins to three other target sequences, AP-1, CREB, and PEBP2, as well as the \textit{in vivo} transcription of SV40 promoter sequences. The inhibitor was also found in UV-irradiated melanocytes, suggesting that it is tissue specific. The induction of a transcriptional inhibitor in response to UV irradiation represents a regulatory event that may play an important role in the transcriptional response of both normal and malignant melanocytes to UV irradiation.

Introduction

UV irradiation plays an important role in the development of human melanoma, a tumor notoriously resistant to radiation therapy (1, 2). In addition to its role in tumor initiation, in which UV irradiation generates characteristic photo adducts that can lead to somatic mutations (3, 4), UV is also a potent tumor promoter. When tested in mouse skin, the tumor-promoting activity of UV irradiation is considerably greater than its initiating activity (5). The response of mammalian cells to UV irradiation has been shown to trigger the activation of Src tyrosine kinases, followed by activation of Ha-Ras and Raf-1 (6).

These changes modify phosphorylation patterns of other target proteins, such as c-jun and NFkB (7, 8), which dictate their transcriptional activities. Among UV-inducible genes are repair enzymes (3), c-myc (9), c-fos (10), the tumor suppressor gene p53 (11), protein kinase C (12), and growth arrest DNA damage genes (13). Together, these genes participate in a mammalian "SOS"-like response. A similar response is elicited by other DNA-damaging agents such as ionizing radiation, alkylating agents, quinones, and hydrogen peroxide (8, 14).

We used the Polyoma virus as a marker gene to study the mammalian response to DNA damage. Polyoma virus undergoes selective amplification (also known as asynchronous viral DNA replication) in response to UV irradiation when present in a semipermissive host cell such as rat fibroblasts (reviewed in Refs. 15 and 16). We have previously demonstrated that the selective amplification of viral genes involves UV-inducible host cell-regulatory factors (17). Damage-inducible cellular proteins have been found to induce similar responses in SV40 (18), adeno-associated helper virus (19), and HIV-1 (20). In characterizing the response of Polyoma to UV irradiation, we have identified a URE (21) which controls transcription and replication of Polyoma virus (22). The URE, with one base pair mismatch, is present in the upstream promoter regions of c-jun (23) and tyrosinase (the key enzyme for melanin synthesis; Ref. 24), and on the long terminal repeat of HIV-1 (25), genes that are expressed at higher levels after UV irradiation (22–25). Proteins that bind to the URE increase their patterns of expression following various stress-inducing treatments to human keratinocytes (26). Several independent studies have identified the proteins that bind to the URE, on the c-jun promoter, to include c-jun itself (23), c-fos (27), ATFII (28), and CREB (29), suggesting that regulation mediated by the URE may involve different proteins in different cell types under various growth conditions.

In the present study, we explore the binding activity of nuclear proteins interacting with the URE in both normal and malignant melanocytes. We demonstrate that UV-irradiated melanocytes and melanoma cells produce an inhibitor which abolishes the ability of nuclear proteins to interact with their target sequences. Such changes in transcriptional activities may represent an important regulatory event which could affect the response of this tissue to UV irradiation and perhaps to other types of stress.

Received 1/27/93; revised 4/1/93; accepted 4/27/93.

1 This study was supported by Grants CA 59908 (Z. R.), CA 51995 (Z. R.), and CA44542 (R. H.) from the National Cancer Institute, SIGBA from the American Cancer Society (Z. R.), and ES 05337 from the National Institute of Environmental Health Sciences (S. E. R.).

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: URE, UV-responsive element; CRE, cyclic AMP-responsive element; CREB, CRE-binding protein; EMSA, electrophoretic mobility shift assay(s); PEBP2, Polyoma enhancer-binding protein 2 (= PEAA2); SOS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; KDa, kilodalton(s); FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol.
Results

Binding of Proteins from Melanocytes and Malignant Melanoma Cells to the URE. In contrast to previous findings with rodent fibroblasts and human keratinocytes, in which significant binding of nuclear proteins to the URE occurred only after UV irradiation (22, 26), melanoma-derived proteins exhibited very strong binding activity to the URE prior to UV irradiation (Fig. 1A). Nuclear proteins of normal melanocytes, on the other hand, demonstrated weak binding to the URE, similar to the levels observed with human keratinocytes or rodent fibroblasts (Fig. 1B). A second group of melanoma cell lines, represented by nuclear proteins from A375 and A2058 (Fig. 1B), also exhibited low basal binding activity to the URE. Competition EMSA, using an excess of unlabeled URE or AP1 sequences, indicated that the proteins that complexed with the URE were distinct in part from those that bound to AP1 (Fig. 1C; Refs. 21, 22, and 26). The similarity between URE and AP1 as well as the complexity of fos and jun protein families suggest that other members of the jun/fos family may participate in this reaction. The strong basal binding activity to the URE observed in 6 of 8 melanoma cell lines may be due to a melanoma-specific posttranslational modification of URE-binding proteins, since the pattern of melanoma nuclear proteins, purified through a URE affinity column or analyzed by Western blotting using antibodies to URE-binding proteins, was similar to that observed with proteins from human keratinocytes (data not shown). Furthermore, in vitro dephosphorylation of melanocyte nuclear proteins with calf intestine alkaline phosphatase enabled strong binding activity, supporting the role of posttranslational modification in the binding activity of URE-bound proteins in normal and transformed melanocytes (data not shown).

Since the binding activity of nuclear proteins to the URE from melanocytes and melanoma cell lines was reduced following UV irradiation (Fig. 1A), we explored the mechanism responsible for the decrease in binding.

UV Irradiation of Melanoma Cells Activates an Inhibitor Which Abolishes Binding of Nuclear Proteins to the URE. To determine whether the decreased binding could be attributed to a UV-inducible inhibitor, nuclear or cytoplasmic proteins from UV-irradiated melanoma cells were incubated with nuclear proteins from unirradiated melanoma cells (MeWo) prior to the addition of 32P-labeled URE to the reaction mixture. As shown in EMSA (Fig. 2), the addition of nuclear proteins from UV-treated MeWo cells completely abolished the strong binding of nuclear proteins from sham-irradiated MeWo cells in a concentration-dependent manner. Whereas complete inhibition was observed at a 1/25 dilution of proteins from UV-irradiated MeWo cells, a significant inhibition was also achieved with 1/125 dilution (Fig. 2). To determine whether this inhibitory activity was also present in the cytoplasm, cytoplasmic proteins were added, in the same dilutions, to the binding reactions. As shown in Fig. 2, the cytoplasmic fraction did not contain the inhibitor. The slight decrease in binding in high concentrations (1/5 dilution) may have been due to the presence of a small amount of nuclear proteins within the cytoplasmic fraction.

Analyzing the binding activity of normal melanocytes has revealed weak binding that became weaker following UV irradiation. To test whether the inhibitor is also present in melanocytes, nuclear proteins prepared from me-
lanocytes prior to and following UV irradiation were mixed with those prepared from 3SS melanoma cells, which exhibit strong basal binding activity to the URE. These experiments revealed that nuclear proteins from UV-treated melanocytes were capable of blocking URE-protein complexes in a fashion similar to that observed with the melanoma-derived proteins after UV irradiation (data not shown), suggesting that inhibitory activity is also present in UV-treated melanocytes. The inhibitor was not present in nuclear proteins from UV-treated human keratinocytes, suggesting expression in a tissue-specific manner.

Binding and Inhibitory Activities Are in Different Protein Fractions. To further characterize the inhibitor, nuclear proteins from UV-treated MeWo cells (which lack binding activity) were fractionated by FPLC (Fig. 3A) using a gel filtration column. Each even-numbered fraction was concentrated (see “Materials and Methods”) and tested for the ability to bind to the URE. Fig. 3B demonstrates that the binding proteins were found in fractions 22–24, which comprised the second of four peaks from the FPLC gel filtration column (Fig. 3A).

To identify the inhibitor, each of the odd-numbered fractions was concentrated, mixed with nuclear proteins from sham-treated MeWo cells, and analyzed in EMSA. This analysis revealed that the inhibitor was present in fraction 47 (Fig. 3C). This fraction is within the last peak of the FPLC gel filtration eluate (Fig. 3A), which consists of low-molecular-weight proteins. The enhanced binding activity in some of the fractions (fractions 21–29 and 39–41; Fig. 3C) may be due to the presence of additional URE-binding proteins which act as synergists.

Identification of Inhibitor Protein. To identify the fraction mediating the inhibitory activity, we iodinated protein samples of fractions 24 and 47, representing binding and inhibitory activities, respectively. Lociation of proteins, followed by SDS-PAGE and autoradiography, showed that fraction 24 consisted of several proteins, including 68 and 40 kDa proteins that we have previously identified as URE-binding proteins in rat fibroblasts (26). That several other proteins were also observed in this fraction is explained by the fact that the separation of these proteins through FPLC analysis was based on protein size rather than binding activity to the URE. The low-molecular-weight protein may represent a subunit or degradation fragment of the higher-molecular-weight proteins present in this fraction. The latter may also be part of a complex formed with one of the higher-molecular-weight proteins noted in this fraction. Analysis of fraction 47, which contained the inhibitory activity, revealed 12 and 14 kDa proteins (Fig. 3D).

We tested whether the inhibitor is heat sensitive to further confirm that it is a protein. To this end, nuclear proteins from IVcl-1 cells were prepared 24 h following UV irradiation and mixed with nuclear proteins from non-UVR-treated cells (1:1 ratio using 7.2 μg proteins from each type) prior to or following their incubation in 65 or 95°C. As shown in Fig. 3E, heating the UV-derived IVcl-1 proteins decreased the inhibitory activity in a temperature-dependent manner.

Specificity of the Inhibitor. We tested whether nuclear proteins from UV-treated 451.LV cells could also modulate the binding of nuclear proteins from sham-treated 451.LV cells to regulatory elements other than the URE. EMSA with DNA fragments containing the binding sites for AP1, CREB, or PEBP2 revealed that most of the complexes formed with the respective transcription factors to each of these sequences were inhibited by these proteins (Fig. 4A), suggesting that the inhibitor may contribute a general effect in transcriptional control.

To test whether the inhibitor is capable of blocking the binding of nuclear proteins prepared from other species, we have used proteins from UV-treated mouse fibroblasts (WOP cell line, which exhibits stronger binding to the URE following UV irradiation; Ref. 26). Indeed, proteins from UV-treated 451.LV cells were capable of blocking binding of mouse proteins to the URE (data not shown). Addition of nuclear proteins from UV-treated human keratinocytes to those of human melanomas did not modify the intensity of the complexes formed with the URE (data not shown). The latter finding is supportive of our previous studies demonstrating that fibroblasts and keratinocytes exhibit stronger binding to the URE following UV irradiation (22, 26).

Mechanism of Inhibitor Activity. In all EMSA performed to this point, we have added the nuclear proteins from untreated cells to those prepared after UV exposure prior to the addition of the ^32P-labeled target sequence to the reaction mixture. It was also of interest to determine whether the inhibitor would be active when added to the reaction following the incubation of nuclear pro-
Fig. 3. Identification of the UV-inducible inhibitor in melanoma cells. Panel A, pattern of fractionation of nuclear proteins from UV-treated MeWo cells using gel filtration FPLC column. Panel B, analysis of FPLC fractions for binding to the URE. Even-numbered fractions were reacted with the \( ^{32}P \)-labeled URE in EMSA. The migration of the free URE is shown in Lane P. Panel C, analysis of FPLC fractions for inhibitory activity. Odd-numbered fractions were incubated with nuclear proteins (7.2 \( \mu \)g) from nonirradiated MeWo cells, and their binding to the \( ^{32}P \)-labeled URE was determined in EMSA. The migration of URE without proteins is shown in Lane P. Panel D, identification of low-molecular-weight proteins in the inhibitor fraction. Induction of fractions 24 and 47 followed by separation on 12% SDS-PAGE and autoradiography revealed the molecular weight of the binding proteins and of the inhibitor (arrowheads). Panel E, the inhibitor is heat sensitive. Shown is EMSA in which \( ^{32}P \)-labeled URE (Lane A) was incubated with nuclear proteins prepared from IVCl-1 cells (Lane B) in the presence of nuclear proteins prepared from UV-treated IVCl-1 cells (Lane C) that were incubated at 65°C or 95°C for 10 min (Lanes D and E, respectively).

teins with the labeled target sequence. To this end, nuclear proteins from 451.LV cells were incubated with a \( ^{32}P \)-labeled URE for 20 min prior to the addition of nuclear proteins prepared from UV-treated 451.LV cells. As shown in Fig. 4B, when the components were added to the reaction mixture in this order, the inhibitor was no longer capable of blocking the binding to the URE. These results suggest that the inhibitor alters the respective transcription factors prior to their interaction with their target sequences.

The Inhibitor Is Active in in Vitro Transcription Assays.

As the inhibitor was capable of blocking the formation of complexes by proteins from mouse cells and those formed with target sequences other than the URE, we also tested whether it would be capable of blocking the in vitro transcription of SV40 promoter sequences mediated by Drosophila embryo nuclear extracts. We chose to use Drosophila embryo extracts since they are known to exhibit strong transcriptional activity. The transcripts produced in the in vitro transcription reactions were determined via primer extension reaction. Whereas a control transcription reaction generated the expected size transcript, the addition of nuclear proteins from UV-treated melanoma cells decreased the quantity of this
transcript in a concentration-dependent manner (Fig. 5). Quantitation of the amount of radioactivity in the different transcripts was performed with the aid of a radioimaging blot analyzer. The decrease in the amount of transcripts was not observed when nuclear proteins from sham-treated melanoma cells were added to the reaction mixture (Fig. 5).

Discussion

A large subset of genes including transcription factors, growth factors, and enzymes involved in signal transduction pathways have been found to exhibit different levels of expression in melanocytes when compared to other tissue types. Yet, the mechanisms which control the changes in the expression of these regulatory genes have not been identified.

We demonstrate here that nuclear proteins from 6 of 8 human melanoma cell lines bind with greater affinity to the URE than those isolated from normal human melanocytes or, as previously shown, from rodent fibroblasts or human keratinocytes. Although only a small fraction of melanoma cell lines were tested, these results suggest that a regulatory mechanism that controls the activities of URE-binding proteins undergoes a significant change during malignant transformation. The increased basal activity of nuclear proteins to bind the URE, in 6 of 8 melanoma cell lines, could result from: (a) posttranslational modifications conferring a constitutive URE-binding activity, and/or (b) the expression of a different subset of proteins which binds with higher affinity. The observation that different transcription factors bind to the URE of the c-jun promoter (23, 27–29) supports the latter hypothesis. Nevertheless, in vitro dephosphorylation studies with nuclear proteins of melanocytes resulted in the acquisition of strong binding activity, whereas the same treatment to melanoma-derived proteins resulted in lower binding activity to the URE, suggesting modification by phosphorylation. Thus, at the present time, supportive evidence for both hypotheses exist.

The proteins from 6 of the 8 melanoma cell lines that bound strongly to the URE were inhibited in their binding following UV irradiation. The decrease in binding to the URE was also noted in UV-irradiated normal melanocytes. This response appeared to be due to a low-molecular-weight inhibitor. The inhibitor also interfered with the binding of nuclear proteins to selected target sequences such as AP1, PEBP2, and CRE, the binding of nuclear proteins from mouse fibroblasts to the URE, and blocked in vitro transcription, suggesting that it is neither species nor sequence specific. It appears that the inhibitor must interact with the binding proteins prior to their interaction with their target sequences. The level of inhibitor activity was found to vary between different cell lines, an observation we presently attribute to posttranslational modification, which differs in these cell lines. It is possible that the inhibitor may exhibit kinase activity which is necessary to allow protein binding to DNA. Partial purification of melanoma nuclear proteins by FPLC demonstrated that the binding and the inhibitor activity were present in different fractions and that the inhibitor is a low-molecular-weight protein (12–14 kDa). That a protein of similar molecular weight was also noted in the binding fraction may suggest that the latter could originally be part of a complex formed with higher-molecular-weight proteins found in this FPLC fraction. Overall, our observations suggest that: (a) the decrease in binding activity in UV-C-irradiated cells is not due to changes in the concentrations of the URE-binding proteins; (b) the inhibitor is UV-induced/activated and is capable of reducing the binding of the nuclear proteins to the URE; (c) the inhibitor activity appears to be nonspecific as it blocked binding of nuclear proteins from different species to different target sequences. It is not likely that the inhibitor is a protease, since it was identified in the nuclear protein fraction, and its effect can be restored as noted in our FPLC analysis, in which total nuclear proteins that did not have binding activity exhibit

* Y. M. Yang and Z. Ronai, unpublished observations.
Effect of the inhibitor on in vitro transcription of SV40 promoter sequences. RNA transcripts were synthesized in vitro from SV40 promoter sequences with the aid of Drosophila embryo nuclear extract in the absence (Dros) or presence of nuclear proteins from control (Dros+Mel-cont) or (Dros+Mel-UV) UV-treated 451.LV cells (in nondiluted and 1/30 and 1/90 dilutions). The size and amount of transcripts were determined using primer extension reaction. The amount of transcript in femtomoles RNA was calculated following quantitation with a radiomaging blot analyzer.

the characteristic binding complexes following FPLC fractionation.

The observation that melanocytes also contain the UV-inducible inhibitor suggests that it may be tissue specific. Although the functional role of the inhibitor in the overall response of melanocytes and melanomas to UV irradiation has yet to be determined, its presence is unique because it does not exist in fibroblasts or keratinocytes. The inhibitor could alter cellular response to UV irradiation as well as other types of stress through its ability to modify activity of transcription factors. The latter could provide the molecular basis for the resistance of melanocytes to UV irradiation.

Materials and Methods

Cell Cultures. Normal human melanocytes were cultured from newborn foreskins in Ham's F-10 medium supplemented with 2.5% fetal bovine serum, 2.5% calf serum, 85 mM 12-O-tetradecanoylphorbol-13-acetate, 0.1 mM isobutylmethyl xanthine, 40 μg/ml pituitary extract, 1.0 mM insulin, 1.0 μM Na2VO4, and 0.1 mM ascorbic acid as described (30). Melanocytes at passages 2–6 were used for these studies. The human melanoma cell lines MeWo, 355, 451.LV, WM 164, IV C1 and, and IV C1-3 were from Dr. C. V. Hamby (31, 32); MeWo, 451.LV, and IV C1-3 are the metastatic forms of 355, WM 164, and IV C1-3, respectively. Melanoma cell line A375 was bought from the American Type Culture Collection, and A2058 was obtained from Dr. Mark Sobel, National Cancer Institute, Bethesda, MD. Melanoma lines MeWo, 355, IV C1-3, and IV C1-3 were maintained in RPMI 1640 supplemented with L-glutamine and 5% FBS; WM 164 and 451.LV in Leibovitz's medium supplemented with L-glutamine and 5% FBS; A2058 in Dulbecco's modified Eagle medium supplemented with 10% FBS and basal amino acids; and A375 in Dulbecco's modified Eagle medium supplemented with 4.5 g/liter d-glucose and 10% FBS.

Oligonucleotide Probes. Oligonucleotides representing a dimer of the URE (ACTATGACAACAGCTATGACACAGT), CRE (ACCATGACGTGACTATGACGTCACTGAT), PEBP2 (ACTGACCCGACGACTGACCGACGCT), or AP1 (CTGACTCATCCGTGACAACT) target sequences were synthesized in-house with the aid of a Cyclone DNA synthesizer (Milligen Biotech). Complementary DNA strands were purified and annealed according to standard procedures.

UV Irradiation, Nuclear Protein Preparation, and Electrophoretic Mobility Shift Assay. Cells were irradiated with UV-C (254 nm) at 12 J/m2 as described (33). This dose caused less than 30% cytotoxicity by 24 h as determined by vital staining. Nuclear and cytoplasmic proteins were prepared from 106 cells 24 h after irradiation or sham treatment as described (34). The fractions were dialyzed, the amount of proteins was determined (BCA; Pierce), and the fractions were stored in aliquots at -80°C.

For EMSA, a dimer of the respective DNA sequence was used as the target for nuclear protein binding. The target sequence was end labeled with a [32P]dCTP and a [32P]dATP (Amersham) using the Klenow fragment of DNA polymerase I (Promega). The nuclear proteins were incubated with a 32P-labeled oligonucleotide as specified in “Results” (0.4 ng, 4000 cpm) for 20 min at 20°C in the presence of 100 ng polydeoxyinosinic-deoxyctydilic acid DNA and DNA binding buffer (10 mM HEPES, 50 mM KCI, 0.1 mM EDTA, 0.5 mM DTT, 4 mM MgCl2, and 10% glycerol) followed by electrophoresis in 7.5% polyacrylamide gel and autoradiography. In competition EMSA, excess of competitor URE or AP1 DNA fragments (as outlined in “Results”) were incubated with nuclear proteins for 10 min prior to the addition of 32P-labeled URE to the reaction.

FPLC Fractionation and Analysis for Binding and Inhibitory Activities. One mg of nuclear proteins from MeWo cells prepared 24 h after UV irradiation was loaded onto a Superose 12 (Pharmacia) gel filtration column and fractionated in the presence of 10 mM HEPES (pH 7.9), 0.5 mM DTT, 0.1 mM EDTA, 4 mM MgCl2, and 200 mM KCl with a flow rate of 0.4 ml/min. Fractions of 400 μl were collected. Even-numbered fractions were concentrated 3-fold with an MC-10 concentrator (10 kDa cutoff; Millipore) and 10-μl aliquots were used in EMSA with 32P-labeled URE to determine the fractions with binding activity. Odd-numbered fractions were concentrated 20-fold by lyophilization and were then used to identify inhibitory activity. Aliquots from each fraction (3 μl) were added to 7.2 μg of nuclear proteins from sham-
treated MeWo cells and subjected to EMSA with $^{32}$P-labeled URE.

Iodination. Aliquots of 25 μl of the specified fractions were iodinated using $^{125}$I (0.1 mCi; Amersham) with the aid of iodobeads (total of 200 μl) for a period of 10 min according to the manufacturer's recommendation; Pierce. Free iodine was removed by dialysis, and trichloroacetic acid (20%)-precipitated materials were loaded onto a 12% SDS-PAGE column, followed by autoradiography.

In Vitro Transcription. A 300-base pair KpnI-HindIII fragment of SV40 promoter region was cloned into pSP72 vector (Promega), flanked by SP6 and T7 promoter sequences. One μg of SV40 promoter DNA was used as template for an in vitro transcription reaction performed in the presence of transcription buffer (7.5 mM HEPEs (pH 7.6), 60 mM potassium glutamate, 3.75 mM MgCl₂, 0.03 mM EDTA, 1.5 mM DTT, and 3% glycerol), 0.5 mM concentration each of rATP, rCTP, rGTP, rUTP, and 20 μg of Drosophila embryo nuclear extract (Promega) in a total volume of 20 μl incubated at 21°C for 60 min. To these reactions, 10, 1, or 0.3 μg of nuclear proteins (in the presence of RNase inhibitor, 5 units of RNasin; Promega) from UV-treated 451.LV cells were added (1 μl) as specified. To assay for the levels of specific mRNA, primer extension reactions were performed using $^{32}$P-labeled primer (GATTATAGCTGACACTATAG) complementary to the SP6 sequences on the pSP72 vector. Fifty fmol of labeled primer were annealed with 5 μl (50%) of the RNA produced in the transcription reaction by heating the mixture in the presence of primer extension buffer [100 mM Tris (pH 8.3), 100 mM KCl, 20 mM DTT, 2 mM concentration of each deoxynucleoside triphosphate, and 1 mM spermidine] for 20 min at 58°C. Subsequently, primer extension reaction was initiated with the aid of avian myeloblastosis virus reverse transcriptase (10 units) in the presence of sodium pyrophosphate (40 mM) incubated at 42°C for 30 min in a total volume of 20 μl. Primer extension reaction was terminated by heating the samples to 75°C for 10 min in the presence of 20 μl of loading dye. Twenty-μl aliquots were then fractionated on an 8% acrylamide-7 M urea gel (16 x 40 cm) using a constant voltage of 250 V. Autoradiography revealed the position of primer-extended transcript. The quantity of each transcript as femtomoles RNA was calculated based on the amount of the specific transcript and input primer as measured with the aid of a radioimaging blot analyzer (AMBIS), using the following formulas: (1) cpm of primer input/[50 fmol x (20x40)] = primer cpm/μmol; (2) [transcript cpm/primem cpm/μmol] x 4 = fmol RNA (the 20/40 factor corrects for the amount of primer loaded onto the gel, and multiplication x 4 corrects for the amount used in the analysis).

Acknowledgments
We thank C. Hamby and M. Sobel, who kindly provided us with the melanoma cell lines, and C. Bascillico for the WOP cells.

References