Transcriptional Inhibition of Ha-ras in Interferon-induced Revertants of ras-transformed Mouse Cells

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ABSTRACT

Elevation in Ha-ras expression, due to transcriptional activation or gene amplification, is associated with oncogenic transformation of NIH 3T3 cells. We have previously shown that murine interferon (IFN-α/β) induced phenotypic reversion of NIH 3T3 cells transformed by long terminal repeat (LTR)-activated c-Ha-ras. The revertants produced decreased amounts of ras-encoded M, 21,000 protein. We have now determined the molecular level at which LTR-ras regulation occurred. Nuclear run-on experiments revealed a selective inhibition of ras transcription in IFN-treated revertants. There was no apparent additional posttranscriptional control by IFN as judged by the unchanged half-life of ras transcripts. Inhibition of ras RNA synthesis was seen only in conjunction with long-term IFN treatment and was limited to the revertants, a population of cells that maintained sensitivity to IFN during the prolonged exposure. The reduction in ras activity appeared responsible in part for the loss of tumorigenicity in treated cells and was stable for several weeks after treatment had been discontinued.

INTRODUCTION

Cellular protooncogenes are implicated in regulation of cell growth and differentiation, and in their activated forms they may lead to neoplastic transformation (1, 2). IFNs3 are multi-potent biological response modifiers with negative growth control and antitumor activities (3, 4). It is of interest, therefore, to examine the interrelationship between IFNs and oncogenes. Several laboratories have reported the inhibition of expression of c-Ha-ras, N-ras, c-myc, p53, v-mos, or v-src oncogenes by IFN (5–14). IFN-mediated inhibition of oncogene expression was associated with reduced cell proliferation (5, 7, 8) or loss of tumorigenicity of cultured cells (10, 14). These in vitro findings suggest that a reduction in the level of a transforming gene product or its function could be a part of IFN activity in vivo.

In our laboratory, we have focused primarily on the regulation of Ha-ras oncogene by IFN. Quantitative changes in Ha-ras expression have been seen in a wide variety of human and animal tumors (15–18). Overexpression of ras can result from gene amplification (19), changes in DNA methylation (20), or from activation by transcriptional enhancer elements (21) among other possible causes. Even in cases involving a mutated Ha-ras gene, a significant elevation in ras expression may still be required for cell transformation (22). If abundance of ras p21 protein is needed for the maintenance of the transformed phenotype of some tumor cells, then a decrease in ras expression could bring about reversion to a less transformed phenotype.

In testing this hypothesis, we have utilized an NIH 3T3 cell line transformed by the human c-Ha-ras-I protooncogene transcriptionally activated by a retroviral LTR (cell line RS485). We have shown before that prolonged treatment of RS485 cultures with mouse IFN-α/β resulted in the emergence of numerous revertant cells (13). The isolated revertants, which formed contact-inhibited cultures and were no longer tumorigenic in nude mice, retained the transforming ras DNA, yet in the presence of IFN produced ras mRNA and p21 at significantly lower amounts than parental RS485 cells (13, 14). In this paper we show that LTR-ras regulation by IFN occurred at the level of gene transcription and that this was highly correlated with phenotypic reversion. Mechanisms that might be involved in the control of oncogene expression in IFN-treated revertants are discussed.

MATERIALS AND METHODS

Cells and Reagents. Cell line RS485 was derived by transformation of NIH 3T3 cells with the 2.9-kilobase Sacl fragment of the human c-Ha-ras-I protooncogene ligated downstream from the LTR of Harvey murine sarcoma virus (21). RS485 revertant clonal line 4C3 and its subclone PR4 have been previously described (13, 23). Cells were propagated in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% heat-inactivated fetal calf serum (Gibco Laboratories), 100 units/ml of penicillin, and 100 µg/ml of streptomycin (Sigma). Mouse L-cell IFN-α/β (a gift from M. Pauker, Medical College of Pennsylvania) was purified on an antibody affinity column to a specific activity of 10⁶ IU/mg of protein. IFN was used throughout the study at a concentration of 200 IU/ml. For inhibition of protein or RNA synthesis, cycloheximide (Sigma) and actinomycin D (Sigma) were used at a concentration of 50 µg/ml and 2 µg/ml, respectively. 5AzadC (Sigma) was freshly prepared in PBS and used as described (23).

Northern Blot Analysis. In all experiments total cytoplasmic RNA was extracted from logarithmically growing cultures according to established procedures (24). RNA (25 µg per lane) was electrophoresed in 1% agarose-formaldehyde gels and transferred onto nitrocellulose paper (24). Blots were hybridized to specific 32P-labeled DNA probes prepared by nick-translation using [32P]dCTP (New England Nuclear; specific activity, 3000 Ci/mm) and washed, and exposed to X-ray film at −70°C in the presence of intensifying screens. For densitometric analysis of the signals, care was taken to scan autoradiographs that were exposed within the linear response range of the X-ray films.

Isolation of Nuclei and Preparation of Nuclear RNA Transcripts. Cells were collected, washed with PBS, and lysed with 3 ml of ice-cold 10 mM Tris-HCl (pH 7.5):10 mM NaCl:1.5 mM MgCl2:0.5 mM dithiothreitol:1% Nonidet P-40 (lysing buffer). The nuclei were pelleted by centrifugation at 1000 x g for 5 min at 4°C. Lysis was repeated, and nuclei were washed with cold 5 mM MgCl2:10 mM Tris (pH 7.4) and repelleted by centrifugation. For nuclear RNA analysis, nuclei were extracted in 4 ml of GI solution, and the resulting solution was heated with a 21-gauge needle. Escherichia coli RNA (1 mg) and C3 (1.6 g) were added, and the solution was layered over a cushion of 1.2 ml of 5.7 M CsCl and 50 mM EDTA (pH 7.5) and centrifuged at 40,000 rpm in a SW 50.1 rotor for 24 h at 20°C. The RNA pellet was resuspended in H2O and extracted as described (24).

Nuclear Run-on Transcription Assay. About 1 x 10⁶ nuclei, obtained as described above, were washed with 25% glycerol:5 mM MgOAc;50 mM Tris-HCl (pH 8.0):5 mM dithiothreitol:0.1 mM EDTA (Solution A), pelleted at 12,000 x g for 1 min, and resuspended in 250 µl of...
Solution A. The run-on transcription assay was performed essentially as previously described (25, 26). Briefly, the reaction was initiated by addition of 300 µl of 1 mM each of ATP, GTP, and CTP; 0.24 mM UTP; 4.4 mM MgOAc2; 1.7 mM MnCl2; 0.2 mM KCl; and 300 µCi of [3H]-UTP (New England Nuclear; approximately 760 µCi/mol) to the nuclei suspension. The reaction was allowed to proceed for 20 min at 24°C and then stopped by addition of 4 volumes of G1 solution. RNA transcripts were purified as above, and recovery of trichloroacetic acid-precipitable radioactivity was determined. All nuclei preparations showed a 70 to 80% inhibition of incorporation of [3H]-UTP in the presence of 1 µg/ml of α-amanitin (Sigma). Purified labeled transcripts were hybridized to excess DNA probes (2 µg) immobilized onto GeneScreen Plus filters by dot blotting, after being linearized by DNase I digestion. The same filter was hybridized consecutively to 32P-labeled transcripts from the different tested cell lines to avoid possible variations between blots.

DNA Probes. The SacI fragment of human c-Ha-ras-J was used to detect ras-specific sequences. The 1.8-kilobase fragment of human c-myc third exon and the 760-base pair chicken actin fragment were from Oncor. The probe for 2-5 A synthetase was a 2.2-kilobase EcoRl fragment of the mouse J-2 clone (27), a gift from B. R. G. Williams, University of Toronto, Canada. The 550-base pair PstI fragment of the H-2Ld complementary DNA clone (a gift from G. Jay, NIH, Bethesda, MD) was used as a probe for the mouse major histocompatibility complex Class I genes.

Antiviral Assay. Cells were seeded in 96-well plates at 3 x 10^4 cells per well in the presence of IFN (2-fold dilutions starting with 1000 IU/ml). Control wells contained cells with no IFN. The following day the VSV was added, and lysis of cells was recorded 24 to 48 h after infection.

RESULTS

Decrease in ras mRNA Induced by IFN is Limited to Revertants. Phenotypic revertant clonal lines, of which 4C3 is a representative, were isolated from RS485 cultures after about 2 mo of continuous treatment with IFN; revertant cells produced lowered amounts of ras mRNA and p21 (13). As an extention of these studies we have now investigated whether the reduction in ras expression is a characteristic of the small population of revertants or a more general response of transformed RS485 cells to IFN treatment. Fig. 1 shows that either short or long treatment with 200 IU/ml of mouse IFN-α/β did not result in a detectable reduction of ras mRNA in RS485 cells, the majority of which (about 99% of the total population) was still transformed. The sensitivity of RS485 cells to the IFN treatment was evident from the induction of the mRNAs for 2-5 A synthetase or H-2 antigens, both IFN-inducible genes (3) (Fig. 1). It appears, therefore, that a significant inhibition of ras by IFN occurred only in cells that reverted to a nontransformed phenotype.

Since prolonged treatment may result in a decrease in cellular responses to IFN (28), we tested the sensitivity of RS485 cells that remained transformed after 3 mo of continuous treatment (either the total population or the isolated clonal lines T1 and T2). The results shown in Fig. 1 indicate that the induction of 2-5 A synthetase and H-2 mRNA in the mixed population of IFN-treated RS485 was less profound after 13 wk of treatment than during the first week. Furthermore, in an antiviral assay, 10 to 20 IU/ml of IFN were sufficient to inhibit lysis of previously untreated RS485 by VSV (multiplicity of infection of 10^-1). In contrast, T1 and T2 were only partially protected with 1000 IU/ml of IFN (not shown). It appears, therefore, that at least some of the RS485 cells have lost sensitivity to IFN during the prolonged treatment. Surprisingly, revertant 4C3 cells maintained their sensitivity to IFN for at least 5 mo of continuous growth in the presence of 200 IU/ml of IFN, as determined by the induction of 2-5 A synthetase (Fig. 1) and by the protection from VSV (multiplicity of infection of 0.1) with 35 to 50 IU/ml of IFN. Inhibition of ras expression and phenotypic reversion may require a sustained response to IFN, since short-term treatment with IFN of susceptible RS485 cells was not associated with any significant effect on ras mRNA or on cell phenotype.

Regulation of ras Expression in RS485 Revertants is at the Transcriptional Level. The reduced ras expression observed in IFN-treated revertant cells could result from: (a) lowered rate of ras transcription; (b) more rapid degradation of the transcripts; and (c) both transcriptional and posttranscriptional processes. To determine the molecular level at which the regulation of ras occurred, we first measured the amount of ras-specific nuclear RNA by dot blot analysis. Nuclei of 4C3 cells contained a 6- to 8-fold reduced level of ras transcripts compared to transformed RS485 or to a nontumorigenic subclone of 4C3 (PR4) that was cultured in the absence of IFN for more than 2 mo (Fig. 2A). The reduced ras RNA in the nuclei suggested that the regulation occurs early after or during transcription. Run-on transcription experiments with isolated nuclei (see “Materials and Methods”) were therefore performed to determine the rate of synthesis of ras RNA. In repeated 20-min pulse studies the amount of ras RNA synthesized by IFN-treated 4C3 cells was found to be significantly less than that made by the untreated 4C3 subclone PR4 or by RS485 (Fig. 2B). The rate of transcription of the actin gene was similar in the cells analyzed (Fig. 2B, lower panel), indicating a differential effect of IFN on gene transcription in 4C3 cells.

The relative stability of ras messages in transformed RS485 and in IFN-treated revertant 4C3 cells was also studied. The
cultures were incubated with actinomycin D to block transcription, and RNA samples were collected at various times thereafter and analyzed by Northern blotting (Fig. 3A). Hybridization with c-myc and actin complementary DNAs provided controls for a very short-lived and a stable mRNA, respectively. Kinetic curves of ras RNA degradation, obtained by densitometric analysis of the Northern blot shown in Fig. 3A, indicated that the stability of ras RNA was comparable in RS485 and IFN-treated 4C3 cells (Fig. 3B). The apparent half-life of the ras transcripts was about 4 h in both cell lines.

In an attempt to explain the nature of the transcriptional regulation of ras in 4C3 cells, we investigated whether de novo protein synthesis was required for the inhibition of ras expression by IFN. When 4C3 cells (cultured in the presence of IFN) were treated with cycloheximide, ras mRNA levels increased about 2.5-fold (Fig. 4). Treatment of 4C3 cells with cycloheximide and actinomycin D, to block DNA transcription together with protein synthesis, still resulted in a 2- to 2.5-fold increase in ras (Fig. 4). This suggested that ras mRNA induction by cycloheximide is likely to be due to stabilization of the transcripts on polysemes. However, similar results were seen in 4C3 cells grown in the absence of IFN for a week (not shown) and in untreated RS485 cells (Fig. 4). Therefore, it appears that we have identified an additional mechanism of control of ras expression not related to IFN activity.

Inhibition of ras Expression was Stable Long after IFN Treatment was Discontinued. Withdrawal of IFN from 4C3 cultures was accompanied by only a slight increase in ras message within 7 days (Fig. 1). The time required for a full restoration of ras expression after withdrawal of IFN varied in repeated studies between 3 wk (29) and 3 mo. An example of a fully restored ras transcription is shown for the 4C3 subclone PR4 (Fig. 2). Heritable inhibition of gene expression can result from methylation of the involved DNA sequences (30). To test whether DNA methylation played a role in the stable inhibition of ras, the 4C3 cells were treated with 5AzadC, a demethylating cytidine analogue (31). 5AzadC (0.1 μM), either in the presence or in the absence of IFN, caused retransformation of about 10 to 30% of the 4C3 cells within 2 wk (as determined by an altered morphology and loss of contact inhibition of growth). Retransformation was associated with restored high levels of ras mRNA (Fig. 5). We have previously shown that 5AzadC does not transform NIH 3T3, nor does it induce ras expression in these cells (23).

Correlation between ras Expression and Phenotypic Reversion. In RS485 cells transformation is associated with overexpression of ras. The levels of ras mRNA and p21 in the revertant 4C3 were lower than in transformed RS485, yet significantly higher than that in control NIH 3T3. We tested whether the partial reduction in ras transcription in 4C3 cells played a role in the process of phenotypic reversion. Indirect evidence suggested that oncogenic transformation may require levels of ras higher than those found in revertant 4C3, since all retransformed...
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Fig. 4. Effect of cycloheximide on the levels of ras mRNA. Cells were incubated for 6 h in medium containing: 1, no addition; 2, cycloheximide (50 μg/ml); 3, actinomycin D (2 μg/ml); 4, cycloheximide (50 μg/ml); and actinomycin D (2 μg/ml). Total cytoplasmic RNA was extracted and analyzed by Northern blotting and hybridization to the indicated 32P-labeled probes.

Fig. 5. Restored ras expression after treatment of revertants with 5AzadC. Northern blot analysis of ras expression in: 1, RS485 cells; 2, 4C3 cells; 3 to 5, 5AzadC-induced 4C3-retransformant clones 2, 3, and 4, respectively. 5AzadC clones 2, 3, and 4 are transformed lines isolated following 5AzadC treatment (0.1 μm) of 4C3 cultures in the presence of IFN. The blot was hybridized with a 32P-labeled probe for Ha-ras.

clones of 4C3, whether spontaneous (lines DT5 and DT7) or 5AzadC induced, exhibited increased production of ras mRNA (Figs. 5 and 6).

The 4C3 retransformants were very sensitive to IFN. In particular, lines DT5 and DT7 showed a reduction in ras expression and phenotypic reversion of about 30% and over 50% of the respective cell population after 3 to 4 wk of IFN treatment. Unlike RS485 in which reversion occurred at a lower efficiency, in the IFN-treated DT5 or DT7 cultures the reduction in ras expression could be detected in the total cell population (Fig. 6) without the need to first isolate the revertant clonal lines. The results obtained with these sensitive cell lines indicate a direct effect of IFN on LTR ras, and a correlation between the reduction in ras expression and phenotypic reversion.

DISCUSSION

There are several indications that quantitative changes in Ha-ras p21 may lead to a shift in cell phenotype. Our studies show that, in NIH 3T3 cells transformed by an LTR-controlled c-Ha-ras-I (cell lines RS485, and subclones DT5 and DT7), a partial decrease in ras expression, following a prolonged treatment with IFN-α/β, correlated with phenotypic reversion. It is likely that the level of ras products in IFN-treated revertants is below that needed for oncogenic transformation, as suggested by the finding that retransformation was consistently associated with restored ras expression. The down-regulation of ras mRNA in IFN-treated revertant cells was the result of a decrease in the rate of ras transcription. Since IFN has been shown to regulate other oncogenes at the posttranscriptional level (17, 20), we tested this possibility but found no indication of such a process. The half-life of ras transcripts (as measured by an actinomycin D chase) was similar in IFN-treated revertants and transformed RS485 cells. Furthermore, a posttranscriptional regulation of ras that requires de novo protein synthesis was not affected by IFN (see Fig. 4).
The inhibition of ras and tumorigenicity was seen in only 1 to 10% of RS485 and only after a prolonged exposure to IFN, a treatment protocol that is frequently associated with cell desensitization due partly to down-regulation of cell surface-localized IFN receptors (28, 32). Using the induction of 2-5 A synthetase RNA and the protection from lysis by VSV as indicators for IFN activity, we have shown that the revertants, unlike the majority of RS485 cells, remained sensitive to IFN over several months of continuous treatment. It is likely, therefore, that the process of reversion required long-term response to IFN and that desensitization prevented the majority of the cell population from undergoing reversion.

One interesting aspect of the transcriptional inhibition of ras by IFN was the rather stable nature of that control, even after IFN treatment of revertants was discontinued. IFNs are known to exert a transient effect on cell phenotype and on the transcription of a variety of genes (28, 33, 34). However, such transient effects are usually associated with short treatments, while our studies involved long-term exposure to IFN. The mechanism of the persistent inhibition of ras transcription in the revertants is not known. DNA methylation is one epigenetic mechanism by which alterations in gene transcription could become imprinted and heritable (30). Stable reduction in ras transcription could be explained if there were changes in the methylation pattern of ras sequences or in genes involved in the regulation of ras. Restriction digest analysis of 4C3 DNA using the methylation-dependent restriction enzymes HpaII, MspI, or Hhal (30) did not show alterations in the methylation of ras or of the controlling LTR sequences. This assay is limited, however, to recognizing only changes in the enzyme-specific sites in LTR ras, while modifications of other sites would not be identified. Alternatively, it is possible that changes in methylation have occurred in sequences (other than LTR ras) that regulate ras transcription. Supporting the hypothesis that methylation might be involved in the control of ras in 4C3 is the finding that a transient treatment of 4C3 with the demethylation drug 5AzadC resulted in elevation in ras mRNA and in retransfection. In addition, the prolonged and varying kinetics by which ras transcription was restored after removal of IFN is typical of the heritable, yet unstable, control of gene expression by DNA methylation (30).

Several other groups have studied the effect of IFN on the expression of cellular or viral oncogenes (for review see Ref. 35). As in our studies, stable phenotypic reversion has been seen in BALB/c cells transformed by Moloney sarcoma virus after prolonged treatment with IFN-α/β (10). The revertants transcribed the v-mos mRNA but did not produce mos-containing virions, suggesting a posttranscriptional inhibition. On the other hand, short exposure of human lymphoblastoid cells to IFN caused a transient inhibition of myc expression; the effect on myc was at the transcriptional (8) or posttranscriptional level (7) and was associated with a reversible inhibition of cell proliferation. Interestingly, in Daudi cells treated with IFN-β, the N-ras mRNA was regulated not only quantitatively, but it was also associated with a decrease in electrophoretic mobility which was indicative of a qualitative change (11). In 4C3 revertants, the electrophoretic mobility of the Ha-ras transcripts was unchanged compared to parental RS485.

The transcription of ras in RS485 and revertant cells is controlled by a retroviral LTR. IFN may act on the LTR control element, thereby inhibiting the expression of a linked oncogene in v-mos transformed cells (36). The reduction in mos mRNA was seen 24 h after treatment with IFN-γ and was followed by a reversible phenotypic reversion. In contrast, LTR ras-transformed RS485 cells did not show any reduction in ras transcripts with IFN-γ. Furthermore, inhibition in RS485 revertants was seen only in conjunction with prolonged IFN-α/β treatment, an inhibitory effect that lasted long after treatment had been discontinued. These differences suggest that the down-regulation of the respective oncogenes by IFN occurs through different mechanisms in the ras or mos transfectants.

What is apparent from the different reports on the mechanism of regulation of oncogenes by IFNs is that this may vary depending on the oncogene and the mode of its activation, the cellular system, the type of IFN used, and the duration of IFN treatment. In many cases inhibition of an oncogene is associated with reduced growth or tumorigenicity. However, previously published results from our laboratory (23) suggested that phenotypic reversion could not be explained by an effect on one oncogene alone. Our current studies therefore are aimed at identifying other genes, the expression of which is modulated by IFN, in an attempt to better understand the mechanisms of IFN-induced reversion.

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