Inhibition of 2-5A Synthetase Expression by Antisense RNA Interferes with Interferon-mediated Antiviral and Antiproliferative Effects and Induces Anchorage-independent Cell Growth

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Abstract
It has been shown previously that the IFN-induced enzyme 2-5A synthetase is sufficient to induce antiviral and antiproliferative effects in transfected cells expressing the protein. In this study, the possibility that this enzyme is also essential in generating these biological activities was investigated. For this purpose, a plasmid, pMSas-NEO, was constructed. This plasmid carries an active neomycin-resistant gene. In addition, it contains a metallothionein promoter fused to an inverted 180-bp fragment derived from the 5’ end of cDNA encoding the 43-kDa isomerase of murine 2-5A synthetase. NIH/3T3 mouse fibroblasts were transfected with the plasmid, about 50 neomycin-resistant clones were isolated, and two, clone 11 and clone 22, were chosen for further studies. One clone transfected only with the neomycin-resistant gene, clone Neo, was used as a control. The results show that in the case of clone 11, the combined treatment of IFN and ZnCl₂ reduced significantly the level of the IFN-induced 2-5A synthetase activity, the amount of the 40-, 43-, and 71-kDa 2-5A synthetase isomers and the level of the 1.7-kb specific RNA transcript. An even stronger effect on these parameters was observed with clone 22. No difference in PKR activity was evident under the same conditions with all three clones tested. The most important, the combined treatment of IFN and ZnCl₂ reversed the IFN-mediated antiproliferative and antiviral activities, as determined by the kinetics of cell growth, thymidine incorporation, cloning efficiency, and infection with mengovirus. Strikingly, the growth of colonies in soft agar were observed in both clone 11 (small colonies) and clone 22 (large colonies) cells, particularly following treatment with ZnCl₂. We conclude that 2-5A synthetase is an essential component in the IFN-induced biological activities and that interference with its function results in anchorage-independent growth of the transfected cells.

Introduction
The 2-5A system is one of the major participants in generating the biological activities induced by IFNs (1, 2). Basically, the system consists of three enzymatic activities. The first constituent activated in this system is 2-5A synthetase. At least four 2-5A synthetase isoenzymes in humans (3, 4) and three in mice (5) have been described. These enzymes are induced by IFN, are activated by double-stranded RNA, and catalyze the conversion of ATP into a series of AMP oligomers linked by an unusual 2′-5′ diester bond (1, 6). The 2-5A oligomers then activate a latent RNase, RNase L, the encoding genes of which (of both human and mouse origin) were recently cloned (7). Although present in significant amounts in untreated cells, this enzyme is further induced by IFN (7). RNase L is responsible for the degradation of single-stranded regions of RNA, particularly at sequences containing UU or UA (8, 9) and is therefore an important regulator of protein synthesis. The third enzyme, 2-5A phosphodiesterase (10), catalyzes the cleavage of 2-5A, thus regulating the entire 2-5A system. Recently, the cloning and initial characterization of a 68-kDa RNase L inhibitor was reported (11), which would consequently be an additional modulator of the 2-5A pathway. Early reports demonstrated that 2-5A synthetase and its enzymatic products are present during differentiation and along the cell cycle. For example, a higher level of 2-5A synthetase activity was observed in confluent or serum-starved versus growing cells. Likewise, in G₀-arrested cells, an increased enzymatic activity was demonstrated (12). In addition, the enzyme has been implicated previously in the regulation of cell growth (13–15) and in the differentiation of several cell systems (16–20). Moreover, the level of RNase L was also reported to increase in growth-arrested cells (21) and induced during cell differentiation (22). A direct proof of the involvement of the 2-5A system in antiviral and antiproliferative effects came from transfection studies. Two early reports demonstrated that transfection of 2-5A synthetase cDNA into hamster or murine cells increased the resistance of the cells to the viral infection (23, 24). In a later study, Rysiecki et al. (25) reported that constitutive expression of 2-5A synthetase cDNA transfected into human glioblastoma cells resulted in a reduced rate of cell growth and in an increase in the resistance of the cells to encephalomyocarditis virus infection. Recently, Hassel et al. (26) showed that introduction into murine cells of a
dominant negative mutant of RNase L lacking the carboxyl-terminal 89 amino acid residues suppressed both the antiproliferative and anti-encephalomyocarditis virus effect of IFN. These results clearly indicate that the 2-5A system plays a pivotal role in the development of the IFN-mediated biological properties. However, it is still not clear whether any isoform of 2-5A synthetase, the enzyme responsible for the activation of RNase L, is essential for the generation of the biological effects. It could be argued that other IFN-induced proteins are sufficient to produce the full extent of these effects.

To study this problem, we designed an expression vector harboring an inverted 180-bp fragment derived from the 5' end of murine 2-5A synthetase L3 cDNA encoding the 43-kDa isoform of the enzyme (14). This sequence is fused to the metallothionein promoter. Thus, upon exposure of cells to ZnCl2, 2-5A synthetase antisense RNA should be accumulated. We demonstrate that the presence of that antisense RNA in mouse fibroblasts down-regulates the expression and activity of endogenous IFN-induced 2-5A synthetase, abrogates the IFN-mediated antiproliferative and antimengivirus properties, and facilitates anchorage-independent cell growth in soft agar.

Results

Transfection of pMSas-NEO into NIH/3T3 Mouse Fibroblasts

The expression vector pMSas-NEO (Fig. 1A) carries the metallothionein promoter fused to an inverted 180-bp fragment derived from the 5' end of L3 cDNA (14) followed by the SV40 polyadenylation sequence (see "Materials and Methods"). pMSas-NEO also harbors the neomycin-resistant gene fused to the early SV40 promoter.

The plasmid was transfected into NIH/3T3 mouse fibroblasts, and 50 G418-resistant clones were isolated and further analyzed. Each clone was treated for 24 h with either 240 IU/ml of IFN-α/β or with 240 IU/ml of IFN and 100 μM ZnCl2. 2-5A synthetase activity was then determined in each cell extract. On the basis of the results obtained, the clones were subdivided into three categories. Fully responsive clones were considered to be those in which the enzymatic activity was reduced by more than 80% in cultures treated with IFN and ZnCl2 (which activates the metallothionein promoter) compared to cultures treated with IFN only. Partially responsive clones were those in which the reduction ranged between 20 and 80%, and the clones that exerted a reduction of less than 20% were designated not responsive. As illustrated in Fig. 1B, 14% of the clones were fully responsive, 68% were partially responsive, and 18% were not responsive (did not respond to ZnCl2). One fully responsive clone (clone 22) and one partially responsive clone (clone 11) were chosen for further studies. In parallel, five NIH/3T3 clones transfected with p19SV-NEO (pUC19 containing only the SV40 promoter fused to the neomycin-resistant gene) were isolated. One (clone Neo) served as a control.

Expression and Activity of 2-5A Synthetase in NIH/3T3 Clones Expressing 2-5A Synthetase Antisense Sequences

To clearly demonstrate the specific reduction of 2-5A synthetase expression and activity in NIH/3T3 clones harboring pMSas-NEO, cultures containing clone 11, 22, and Neo cells were treated for 24 h with either ZnCl2, IFN, or both (IFN + ZnCl2). One set of cultures remained untreated. Cell extracts were prepared, and 2-5A synthetase activity was determined in each extract. The 2-5A oligomers synthesized in the reaction were separated on TLC sheets. The appropriate spots are presented in Fig. 2A, and the radioactivity present in each spot is illustrated in Fig. 2B. The results show that no significant level of enzymatic activity could be detected in cell extracts prepared from either untreated or ZnCl2-treated cultures. On the other hand, all clones responded in an elevated level of 2-5A synthetase activity following exposure to IFN (Fig. 2B, lanes 3, 7, and 11). However, whereas in clone Neo, a combined treatment of IFN and ZnCl2 did not alter the level of activity compared to cultures treated with IFN only, it reduced the level of activity by 34% in clone 11 cells and by over 90% in clone 22 cells under the same conditions. This is a clear indication of the ability of 2-5A synthetase antisense sequences to interfere with the appearance of enzymatic activity in transfected clones.

Our 2-5A synthetase enzymatic assay does not differentiate between the various isoforms of the enzyme. To clarify which isoforms are affected by the presence of the antisense sequences in transfected clones, immunoblot analysis was performed on cell extracts of each of the three clones men-
tioned above, using antibodies directed against an epitope common to all forms of 2-5A synthetase (fragment B). As expected, only a minor band of 43 kDa could occasionally be observed in extracts prepared from untreated or ZnCl₂-treated cultures of all clones tested (Fig. 3A). In contrast, in IFN-treated cultures, three bands of 40, 43, and 71 kDa were visible in all the clones tested (including clone 22, in which a faint band of 71 kDa was observed; Fig. 3A, lanes 3, 7, and 11). In the experimental system used in this study, we could not detect the 102-kDa isoform (5, 14) in any of the clones used. The dramatic reduction in the amount of 2-5A synthetase proteins in the transfected cells is clearly demonstrated in cultures treated with both IFN and ZnCl₂ (Fig. 3A, lanes 4, 8, and 12). Whereas practically no reduction in the level of proteins compared to cultures treated with IFN alone was observed with clone Neo cells [Fig. 3B, compare lanes 3 and 4 and see quantitation of 43-kDa isoforms (p43)], a partial reduction was visible in clone 11 cells (Fig. 3, A and B, lanes 7 and 8) and a complete reduction was obtained with clone 22 cells (Fig. 3, A and B, lanes 11 and 12). This is in agreement with the results observed with the enzymatic activity (Fig. 2). It should be noted that the reduction in the amount of 2-5A synthetase proteins was evident with all the three isoforms detected in our analysis, including the 71-kDa band, indicating that the antisense sequences present in the cells interfere with the appearance of all of these isoforms.

The data obtained thus far could be interpreted as a direct effect of the 2-5A synthetase antisense RNA on translation of the appropriate endogenous mRNA molecules. However, the possibility that, in addition, these mRNA molecules are susceptible to regulation by double-stranded RNases present in the cells (27) must be studied. For this purpose, a Northern blot analysis was performed on total RNA extracted from the

three clones used in this study, treated either with IFN or with IFN + ZnCl₂. As a probe, the L3 cDNA encoding the 43-kDa isoform (14) was used. A specific band of 1.7-kb representing a 2-5A synthetase specific transcript was detected in all cultures treated with either IFN or IFN + ZnCl₂ (Fig. 4A, lanes 3, 4, 7, 8, 9, 11, and 12). No detectable amounts of transcripts were observed in untreated or ZnCl₂-treated cultures (Fig. 4A, lanes 1, 2, 5, 6, 9, and 10). The results further show that...
a slight decrease in the amount of 2-5A synthetase-specific transcripts was monitored in clone Neo cells treated with IFN + ZnCl₂ versus cells treated only with IFN (Fig. 4, A and B, compare lanes 3 and 4). This might be due to a general toxicity of Zn²⁺ ions. However, a more profound reduction in the level of 2-5A synthetase RNA was observed under the same conditions with clone 11 (Fig. 4, A and B, compare lanes 7 and 8). The level was further reduced in clone 22 cells (Fig. 4, A and B, compare lanes 11 and 12). In no case was the reduction complete, and even in the latter clone, detectable amounts of transcripts were still visible. This is in contrast to the amount of 2-5A synthetase proteins that were completely abolished in clone 22 cells treated with IFN + ZnCl₂ (Fig. 3, A and B).

We conclude that in transfected clones that are treated with ZnCl₂ (and hence express the 2-5A synthetase antisense sequence), reduction in 2-5A synthetase proteins is a result of interference with both the amount and translation of mRNA molecules. An additional interesting observation is that in IFN-treated transfected clones, particularly in the case of clone 22 cells, the level of 2-5A synthetase activity, 2-5A synthetase proteins, or 2-5A synthetase-specific RNA transcripts was lower than that detected in clone Neo cells (Figs. 2, 3, and 4, compare lane 11 with lane 3). This is most likely due to the "leakiness" of the metallothionein promoter in these clones.

**Specificity of Interference by 2-5A Synthetase Antisense RNA**

It could be argued that the reduction of 2-5A synthetase specific RNA transcripts and proteins in the transfected cells is a manifestation of a general inhibitory effect of antisense sequences on gene expression in these cells. To exclude this possibility, we determined the activity of another IFN-induced protein, PKR, in the transfected clones. The gene encoding mouse PKR has been cloned and in the promoter region, several motifs common to other IFN-induced genes, including 2-5A synthetase, were detected (28). We thought, therefore, that PKR would be the best candidate for testing the specificity of the antisense RNA effect on target genes. Cell extracts were prepared and PKR activity was determined by SDS-PAGE analysis. The presence of the autophosphorylated form of PKR with a molecular mass of 68 kDa as an indication of PKR activity is presented in Fig. 5A, and the quantitation of the bands by densitometry is shown in Fig. 5B.

It is obvious that PKR activity was detected only in cultures treated with IFN or IFN + ZnCl₂ and that in all clones tested, no reduction in enzymatic activity was observed in cultures treated with IFN + ZnCl₂ versus those treated with IFN alone (Fig. 5, A and B, compare lanes 3 and 4, 7 and 8, and 11 and 12). We conclude that the 2-5A synthetase antisense RNA sequences most likely interact specifically with endogenous 2-5A synthetase mRNA molecules.

**Effect of 2-5A Synthetase Antisense RNA on IFN-induced Biological Activities**

**Antiproliferative Effect.** Following the observations that the expression of 2-5A synthetase antisense RNA in transfected cells aborts the appearance and enzymatic activity of the endogenous enzyme, it was essential to study whether this phenomenon has any impact on the IFN-mediated biological activities. First, we tested the antiproliferative effect. Cultures were treated with 1200 IU/ml of mouse IFN-α/β and viable cell counts were performed at daily intervals. The results are presented in Fig. 6A. The addition of ZnCl₂ to untreated clone Neo (control) cells did not alter the kinetics of cell growth. However, IFN treatment reduced significantly the growth rate, which was not affected by the combined treatment of IFN and ZnCl₂, indicating that, as expected, the salt did not impose any nonspecific effects on the growth of NIH/3T3 mouse fibroblasts. In contrast, addition of ZnCl₂ to untreated clone 11 cells reduced their growth rate to some extent. IFN treatment, on the other hand, had a profound effect on the growth rate of these cells. The antiproliferative activity was considerably reversed when the cells were treated with both IFN and ZnCl₂. The best example for the effect of antisense RNA on the manifestation of the IFN-induced antiproliferative property is demonstrated in clone 22 cells (Fig. 6a, panel C). ZnCl₂ alone did not alter their growth rate, whereas IFN alone showed a dramatic inhibitory effect. However, the combined treatment of IFN and ZnCl₂ completely reversed the inhibition. These results are supported by those obtained with the study on thymidine incorporation (Fig. 6b). Here, to monitor the early effects, IFN, ZnCl₂, or both were added for 24 h, and thymidine incorporation lasted for 1.5 h. The results clearly show that although the combined treatment of IFN and ZnCl₂ did not reverse the inhibition observed with clone Neo cells treated with IFN alone, almost complete recovery was obtained with clone 11 cells, and complete recovery was monitored with clone 22 cells under the same conditions, indicating that only in the
transfected clones was a recovery of the IFN-induced anti-proliferative property evident.

To further support this conclusion, we used a third biological parameter, the cloning efficiency of the various cells, as an indication for the ability of the cells to replicate and form a colony. The cells were plated at 200 cells per plate and after 24 h were treated with IFN, ZnCl₂, or both. The cultures were fixed and stained 10 days later. As shown in Fig. 7, addition of ZnCl₂ alone had no effect on the cloning efficiency of any of the clones tested (see Fig. 7B, columns 1, 2, 5, 6, 9, and 10). Addition of IFN alone reduced the cloning efficiency by 30–40% (Fig. 7B, columns 3, 7, and 11). Treatment with both IFN and ZnCl₂ did not alter the cloning efficiency of IFN-treated cloneNeo cells (Fig. 7B, column 4); however, it increased the cloning efficiency of clone 11 cells to almost the control level (Fig. 7B, column 5) and completely reversed the inhibitory effect in the case of clone 22 cells (Fig. 7B, column 12).

These results are in complete agreement with those obtained with both the kinetics of cell growth (Fig. 6a) and thymidine incorporation (Fig. 6b).

**Antiviral Effect.** Because 2-5A synthetase has been shown to be involved in antiviral activity (23–25), we explored the possibility that expression of the antisense RNA sequences will result in at least partial abolishment of this IFN-mediated biological effect. For this purpose, cultures (in 24-well plates) were treated as indicated and infected with mengovirus. Part of the cultures remained uninfected. The cultures were then fixed and stained. Uninfected cells of all three clones grew to a similar density following all treatments. For convenience, only the wells containing cloneNeo cells are presented in Fig. 8 (no virus). The results further demonstrate that in the transfected clones, particularly in clone 22 cells, a combined treatment with 240 IU/ml IFN and ZnCl₂ increased the sensitivity of the cells to virus infection, as indicated by a severe cytopathic effect observed in this culture (Fig. 8, bottom row), compared to a more protected layer of cells treated with IFN only (Fig. 8, third row). We conclude that expression of 2-5A synthetase antisense RNA partially abolishes the IFN-induced antimeangovirus activity.
Expression of 2-5A Synthetase Antisense RNA Facilitates Cell Growth in Soft Agar

In the course of our study, we noticed that in clone 22 cultures, some foci of cell overgrowth were clearly visible although the foci did not cover the entire dish. To clarify whether the abrogation of 2-5A synthetase activity will result in alterations in the growth properties of transfected cells, we tested the ability of the cells to grow in soft agar, a property characteristic of transformed cells. For this purpose, 200 cells, untreated or treated for 24 h with ZnCl₂, were seeded in soft agar, and the colonies developed were scored after 10 days. We could not detect any colonies in clone Neo cells. In contrast, 5 colonies were counted in untreated clone 11 cells and 10 in ZnCl₂-treated cells. However, they were small, and even further incubation did not significantly increase their size (Fig. 9A). In clone 22 cells, on the other hand, about 20 large colonies in the case of untreated cells and 70 similar colonies of ZnCl₂-treated cells developed under the same conditions. A typical colony is presented in Fig. 9B. These results support our earlier conclusion on the weakness of the metallothionein promoter, which allows some expression of the antisense sequences even in cultures not treated with ZnCl₂. It should be noted that we were not able to further expand the growth of the colonies. Removing a colony to liquid medium for growth and expansion resulted in deterioration of the rate of cell growth and eventually to cell death.

Discussion

The data presented in this study clearly demonstrate the significance of 2-5A synthetase proteins as essential elements in the development of the IFN-induced antiproliferative and antiviral activities. Using the antisense RNA strategy to down-regulate the amount and enzymatic activity of 2-5A synthetase and using three different biological parameters, we were able to show that this abolishment of activity results in the interference with the IFN-induced antiproliferative property. Furthermore, under the same conditions, a reduction in the protective effect of IFN against mengovirus infection was clearly visible.

The use of antisense technology for the specific interaction, and consequently for inhibition of expression of the target gene, has been widely exploited in the last decade. This technology has been used to study cancer (29–32), plant (33), cell growth associated (34, 35), and viral (36, 37) genes, among others. The presence of natural antisense RNA molecules encoded by specific genes has been described for prokaryotes, in which it has been shown to regulate the replication of plasmids like ColE1 or FII and other regulatory proteins (37, 38). In addition, similar genes were also reported to function in C. elegans (36, 37). It is therefore obvious that the use of antisense RNA is not only an artificial phenomenon and does occur under natural conditions also.

Our results suggest that the antisense RNA molecules operate on at least two levels. First, it is clear that the amount of 2-5A synthetase-specific transcripts was significantly reduced in transfected clones exposed to ZnCl₂ (Fig. 4). This could be due to either a decreased rate of transcription or an increased rate of mRNA breakdown. Although our experiments do not allow us to differentiate between these possibilities, it should be pointed out that the involvement of RNase H and other RNases in the mechanism leading to antisense RNA activity has been offered for several systems (34, 36, 39). Second, the antisense RNA seems to interfere also with the translation machinery. This is evident from the observation that even with clone 22 cells, which are more susceptible to the effect of antisense RNA, 2-5A synthetase-specific transcripts were still detectable (Fig. 4), whereas no traces of either 2-5A synthetase proteins or enzymatic activity were seen under the same conditions (Figs. 2 and 3). We used in our study a 180 base-long antisense transcript complementary to the 5' region of the 2-5A synthetase mRNA and spanning the RNA translation initiating AUG codon. This approach appears to be more specific than using the entire mRNA complementary strand as has been shown for additional genes (31, 40, 41) and can even be used for the detection of point mutations in susceptible genes (30).

It is interesting to note that although the antisense sequence used in this study was the inverted fragment of the 5'
end of mRNA coding for the 43-kDa isoform of 2-5A synthetase (14), the 40-, 43-, and 71-kDa isoforms were all affected by its presence. This is an indication of the presence of identical or highly homologous sequences at the 5' end of mRNA molecules coding for these proteins. Indeed, a 51% homology in amino acid sequence of the NH$_2$ domain between the human 69- and 40-kDa 2-5A synthetase isoforms was demonstrated (4). Furthermore, several clusters of 15–30 amino acids with a homology of 75–90% were highly conserved (4). In any case, our results do not allow us to attribute a specific biological activity to a particular isoform of 2-5A synthetase. However, in a previous study, it has been reported that a single cDNA encoding the 43-kDa isoform of human 2-5A synthetase was sufficient to confer both resistance to viral infection and growth retardation to transfected cells (25).

The most striking phenomenon observed in this study was the facilitation of anchorage-independent growth of the transfected cells. The number and size of the colonies grown in soft agar in the case of clone 22 were greater than those observed with clone 11 cells. This is in full agreement with the fact that clone 11 belongs to the group of partially responsive clones (Fig. 1B) as indicated by all the parameters used to characterize the clones. In contrast, clone 22 is a fully responsive clone, apparently as a result of highly expressed antisense sequences, and hence a complete reduction of 2-5A synthetase protein was evident with this clone (Figs. 2 and 3). This conclusion is further supported by the results obtained with the clones not treated with ZnCl$_2$. The response of clone 11 cells to the induction of 2-5A synthetase expression and activity by IFN was less pronounced compared to clone Neo cells. The IFN effect was even less effective on clone 22 cells (see Figs. 2–4). This is most likely due to the leakiness of the metallothionein promoter in the transfected clones and indicates that clone 22 expresses the antisense sequences more efficiently than clone 11 cells. Another proof of this suggestion is the fact that even untreated clone 11 or 22 cells produced colonies in soft agar, the latter being, again, more efficient in this respect.

The cell viability of the colonies grown in agar was lost upon transfer and further culturing, possibly as a result of the presence of the inducible promoter used in this study. We are now trying to use a constitutive promoter fused to the antisense construct for the generation of more stable clones. Still, our results clearly indicate that 2-5A synthetase falls into the category of negative cell growth regulatory genes, a notion that has been previously suggested for the IFN system in general (42). Indeed, several reports support this notion. Overexpression of the IFN regulatory factor 1 (IRF-1), one of the major transcription factors involved in the induction of both the IFNs and the IFN-induced genes (42), causes cell growth arrest and chromosomal deletions of IRF-1 is associated with some leukemias (43). On the other hand, overexpression of IRF-2, a repressor that competes with IRF-1 for binding to DNA, results in cell transformation. Thus, the balance between these two factors is the basis for growth control in this case (44). Recently, PKR has also been directly involved in cell growth regulation. It has been demonstrated that two different negative dominant mutants of PKR with alteration in their catalytic domains were able to induce cell transformation in transfected NIH/3T3 cells (45, 46), and a more detailed analysis on the PKR domains responsible for this biological property has been reported (47). In addition, the antisense RNA strategy has also been used for the inhibition of PKR activity, impairing the inducibility of IFN-α and IFN-β in these cells and increasing their permissiveness to viral infection (48). Finally, RNase L is an additional enzyme implicated in the regulation of cell growth, because a negative dominant mutant of this protein interfered with both IFN-mediated antiviral and antiproliferative activities (26). Moreover, the recently discovered RNase L inhibitor (11)

Fig. 9. Growth of colonies in soft agar. Cultures containing clone Neo, clone 11, and clone 22 cells were treated with ZnCl$_2$ for 24 h or remained untreated. About 200 cells from each dish were then seeded in soft agar. Colonies were observed 10 days later. No colonies grew in either treated or untreated clone Neo cells. A, a typical colony of ZnCl$_2$-treated clone 11 cells; B, a typical colony of ZnCl$_2$-treated clone 22 cells. ×250.
further demonstrates the significance of the 2-5A pathway in the regulation of these biological activities.

On the basis of the data presented in this study, we conclude that 2-5A synthetase, the first enzyme activated in the 2-5A cascade, is an essential component of the IFN-mediated antiproliferative and antiviral properties and that the lack of its activity severely abrogates these biological effects. In addition, the enzyme appears to belong to the group of negative cell growth regulatory proteins, the lack of function of which correlates with the appearance of the transformed phenotype.

Materials and Methods

Cell Cultures and Treatments

NIH/3T3 mouse fibroblasts and clones were maintained in DMEM supplemented with 10% newborn calf serum (Biological Industries, Beth Haanek, Israel; complete medium). The cells were routinely cultured in 10-cm tissue culture dishes (Coming, Inc., Corning, NY). Every 3–4 days, the cells were removed with a solution of 0.25% trypsin and 1 ml EDTA in PBS, resuspended in complete medium, and reseded at a ratio of 1:7. The cultures were kept in a humidified incubator flushed with 7% CO2 at 37°C. For all experiments (unless otherwise specified), cells were seeded at 1.0 × 10^5 cells per 10-cm tissue culture dish, and 24 h after, the medium was replaced with fresh medium supplemented with the material mentioned for each experiment. At the appropriate time, cultures were washed twice with PBS, and the cells removed with a rubber policeman into PBS, washed once more, and subjected to analysis as indicated for each experiment.

Mouse IFN-α/β was used in all the appropriate experiments (Lee Biomolecular, San Diego, CA). The specific activity was 9.8 × 10^6 IU/mg. In most experiments, IFN was added at 240 IU/ml (unless otherwise stated). A stock solution of 100 μg ZnCl2 (Sigma Chemical Co., St. Louis, MO; cell culture, catalog number Z-0152) in 50 ml HEPES (pH 6.0) was freshly prepared once a month and stored in small aliquots at −20°C. Treatment of cultures was with a final concentration of 100 μg.

Plasmids

a) pMSas-NEO: the coding sequence for the 43-kDa isoform of murine 2-5A synthetase with an addition of 20 bp upstream from the translation start site (5'-end of the entire fragment) was excised from L3 cDNA (49) with EcoRI. The fragment was ligated to the eukaryotic expression vector pECE (50), which harbors the SV40 promoter and polycydenylation sequence. This ligation resulted in two possible orientations: the sense orientation, in which the 5'-end of the 2-5A synthetase cDNA follows the SV40 promoter, and the antisense orientation, in which the 3'-end of the 2-5A synthetase follows the SV40 promoter. The construct harboring the antisense orientation was truncated by digestion with DraII followed by mung bean nuclease and a second digestion with Smal. Self ligation of this plasmid yielded the final length of 180 bp antisense fragment that included an inverted sequence of the 5'-end of L3 cDNA, as described above. This fragment was excised from the pECE vector with the polycyclenelation signal by double digestion with Bgl II and Sphi and ligated to a vector that harbors the ZnCl2-inducible metallothionine promoter HMT Ix. Finally, the entire HMT Ix fused antisense fragment was inserted into the NdeI site of pUC19, which also contains an active and constitutively expressed neomycin-resistant gene (Fig. 1A). b) p195SV-NEO: this control pUC19 plasmid contains the active neomycin-resistant gene alone.

Transfection

NIH/3T3 fibroblasts were used as host cells. Transfection was performed by the calcium phosphate method as previously described (51). After 48 h of incubation in complete medium, the cultures were subdivided at a ratio of 1:10, and 24 h later, G418 (Calbiochem-Novabiochem Corp., La Jolla, CA) at 800 μg/ml was added. The medium with the drug was replaced once a week. After an additional 14 days, most of the cells died and single colonies were visible. About 50 clones from cultures transfected with pMSas-NEO and 5 from those transfected with p195SV-NEO were carefully removed by trypsin-EDTA solution, resuspended in complete medium with G418, and expanded. Each clone was individually analyzed as indicated in "Results."

Preparation of Cell Extracts (S-10) for Enzymatic Determination

Cell extracts were prepared after the appropriate treatment by removing the cultured cells with a rubber policeman in PBS. The cells were then centrifuged at 800 g in an ice-cold tysis buffer containing 20 mM HEPES (pH 7.5), 5 mM Mg acetate, 0.5% NP40, 1 mM DT, 10% glycerol, and 1 mM EDTA. The extracts were centrifuged at 10,000 × g for 10 min, and the soluble fractions (S-10) were stored at −70°C until use.

Determination of 2-5A Synthetase Activity

Samples containing 20 μg protein from cell extracts in a total volume of 20 μl were mixed with an equal volume of poly(l)poly(C)-agarose beads (Pharmacia, Uppsala, Sweden) prewashed several times with buffer A lacking NP40. The mixture was incubated for 30 min at 30°C, and the beads were then washed several times with the washing buffer. A total of 25 μl reaction buffer was then added containing 20 μM magnesium acetate, 1.5 mM ATP, 1 mM DT, 25 mM HEPES (pH 7.5), 50 μg/ml poly(l)poly(C), and 1 μCi [α-32P]ATP (specific activity, 3000 Ci/mmole; Amersham International, Amersham, United Kingdom). For incubation for 15 min at 30°C, the beads were the sedimented, the reaction buffer containing the enzymatic products was removed, and 20-μl samples from this buffer were incubated with 25 units/ml bacterial alkaline phosphatase (United States Biochemical Corp., Cleveland, OH) for 2 h at 37°C in the presence of 10 μM Tris (pH 8.2). Aliquots containing 3–5 μl each were then placed on polyethyleneimine-cellulose TLC sheets (Merck, Darmstadt, Germany). The sheets were immered in running buffer containing pyri-/acenetic acid/H2O at a ratio of 1:10:100. Chromatography proceeded for 4–6 h. One sample containing 0.05 absorbance unit of authentic forms (2'-5'αA and 2'-5'βA (Pharmacia) was analyzed in parallel, and the spot after migration was identified by illumination with a UV-25 lamp. Its position is marked "2-5A oligomer" in Fig. 2. The sheets were dried and exposed to autoradiography using Agfa Curix RP2 X-ray film.

Determination of PKR Activity

Cell extracts (S-10) were prepared as described above. Heparin (50–100 units/ml) was added to samples containing 500 μg protein each. The mixtures were incubated at 4°C for 10 min. An equal volume of poly(l)poly(C)-Sepharose beads was added at room temperature for 30 min with occasional gentle mixing. The beads were washed several times with buffer B (containing 50 mM KCl, 2 mM Mg-acetate, 7 mM 2-mercaptoethanol, 20% glycerol, and 10 mM HEPES (pH 7.6)) and then once in buffer C (buffer B supplemented with 5 mM MnCl2). The final pellet was resuspended in buffer C supplemented with 1 μCi [α-32P]ATP (50–100 Ci/mmol) and incubated for 30 min at 30°C. After centrifugation, the pellet was washed three times with buffer C and resuspended in 1/10 volume buffer C and 1/10 volume electrophoresis sample buffer containing 6% SDS (w/v), 30% glycerol (w/v), 0.02% bromophenol blue (w/v), 200 μM TRIS-HCl (pH 6.8), and 250 μM 2-mercaptoethanol. The supernatants were collected and analyzed on 10% polyacrylamide slab gels containing SDS. The gel was dried and the phosphorylated proteins were detected by autoradiography on Kodak XAR-5 film. Scanning of the radioactive bands was performed with a GS300 densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Identification of 2-5A Synthetase Isoenzymes by Immunoblotting

The identification of 2-5A synthetase isoenzymes in cell extracts was performed by Western blotting analysis. Samples containing 40 μg of protein were suspended in 30 μl of 0.5 μl Tris-HCl (pH 6.8), 10% glycerol, 10% SDS, 0.05% 2-mercaptoethanol, and 0.05% bromophenol blue, boiled for 5 min and loaded on 10% polyacrylamide-SDS gel. As molecular weight markers, we used the rainbow-colored protein molecular weight markers supplied by Amersham International. Electrophoresis was in the cold at 50 V for 15 min and 200 V for another 1 h. Transfer to nitrocellulose sheets was performed in the minitrans-blot cell (Bio-Rad Laboratories, Hercules, CA) at 4°C in a buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol for 1 h at 200 mA. The nitrocellulose sheet was immersed in blocking solution containing 2% BSA and 0.02% Tween-20 in PBS at 4°C. It was then transferred to
blocking solution supplemented with 1:500 dilution of rabbit polyclonal antibodies directed against fragment B common to all isofoms of 2-5A synthetase (3), incubated for 2 h at 37°C, and then washed three times with 0.02% Tween-20 in PBS. As a secondary detection antibody, we used peroxidase-labeled antirabbit antibodies, and detection was performed by the enhanced chemiluminescence Western blotting procedure as described by the supplier (Amersham). Light emission was detected by a 5-min exposure to Fuji RX Medical X-ray film. Scanning of the 43-kDa band was performed by the GS300 densitometer.

Detection of 2-5A Synthetase-specific RNA Transcripts

For each treatment, three 10-cm tissue culture dishes were used. Total RNA was extracted with tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the protocol supplied by the manufacturer. Samples containing 30 μg of RNA were analyzed on 1% agarose gels in running buffer containing formaldehyde followed by blotting onto nitrocellulose membrane filters (NitroPlus, Micron Separations, Inc., Westboro, MA) as described (52). The ethidium bromide-stained 18S and 28S bands of RNA in each lane were detected on the filters by UV light. No significant differences in intensity between the lanes were observed. For hybridization, the nitrocellulose filters were first prehybridized overnight at 67°C in a solution containing 0.5 N maleic acid, (pH 7.2), 1-mM crystalline bovine serum albumin, 0.5% SDS, (w/v), and 7% SDS (w/v). Hybridization was performed at 67°C for 24 h in the same buffer containing 1×10^6 cpm/ml of 32P-labeled probe. At the end of the hybridization period, blots were washed twice with 1× SSPE (15 mM NaH2PO4, 150 mM NaCl, and 1 mM EDTA) and 0.5% SDS at room temperature for 30 min each and twice with 0.1× SSPE and 0.1% SDS at 60°C for 30 min each. Filters were then exposed for autoradiography. Scanning of radioactive bands was performed with the GS300 densitometer.

Probe

A mouse cDNA clone (E-Mol-3) isolated by J. Chebath and E. M. Coccia from the Weizmann Institute of Science (Rehovot, Israel), was used. The L3 insert is a 1.3-kbp fragment encoding active mouse 43-kDa isoform of 2-5A synthetase and representing a 1.7-kb transcript (14). Following digestion with EcoRI, the fragment was isolated on 1% agarose gel, and about 0.1 μg was labeled with [32P]dCTP (specific activity 3000 Ci/mmol, Amersham) using the rapid multiprime DNA labeling kit as recommended by the supplier (RAN 1601; Amersham). The specific activity was 2–8×10^6 cpm/μg.

Determination of Biological Parameters

Growth Rate. Cells of the different clones were seeded at 2×10^5 cells per 5-cm tissue culture plate and treated 3 h later as indicated in each experiment. For the determination of biological parameters, IFN was used at 1200 IU/ml. At the appropriate times, groups of three plates per point were collected, the medium was removed, the plates were washed twice with PBS, and the cells were collected with trypsin-EDTA solution and resuspended in complete medium. One volume of cell suspension was mixed with an equal volume of 0.4% trypsin blue in normal saline (Life Technologies Inc., Grand Island, NY). Vital cells were counted in a hemocytometer, at 5 different fields per count. The SD of all the counts per time point (total of 15 counts) was determined.

Thymidine Incorporation. Cultures were prepared as described in "Growth Rate." After 24 h of treatment, the medium was removed from the plate and replaced with fresh medium containing 1 μCi/ml of [3H]thymidine (Rotem Industries, Ltd., Beer-Sheva, Israel; 51.6 Ci/mmol) for 1.5 h. The cultures were then washed three times with cold PBS. The cells were lysed with 1% SDS for 10 min at 37°C and the lysates were removed to test tubes. An equal volume of 20% trichloroacetic acid was added, and the tubes were kept on ice for 20 min. The samples were then filtered through Whatman 25-mm GF/c filters (supplied by Tamar, Ltd., Jerusalem, Israel). The filters were dried, placed in toluene-based scintillation fluid, and counted in a Packard 160TR liquid scintillation analyzer. Each point represents the average of three different measurements.

Cloning Efficiency. The appropriate cells were seeded at 200 cells per 5-cm tissue culture plate. One day later, the medium was replaced with fresh medium containing the indicated materials. Groups of three plates were used for each treatment. After 10 days, the medium was removed and the cultures washed once with PBS, fixed with absolute ethanol, and stained with a 1:5 dilution of Giemsa stain.

Growth in Soft Agar. The different cell clones were seeded at 5×10^5 cells per 5-cm tissue culture plate, and 24 h later, the medium was replaced in one group of cultures with fresh medium containing 100 μM ZnCl2. In the second group, the medium was replaced with complete medium only. After additional 24 h, the cells were removed with trypsin-EDTA and resuspended at 200 cells per 5 ml of complete medium containing 0.37% noble agar (Bacto Laboratories, Detroit, MI). The cell suspension was then poured over a 5-mm base layer of solidified complete medium containing 0.5% noble agar in 5-cm tissue culture plates. Three plates per sample were used. The cultures were incubated for 10 days, and the number of colonies was determined in a light-inverted microscope.

Determination of Anti-Murine Virus Activity. For these experiments, 24-well plates were used. Cells were seeded at 2×10^5 cells per well. After 24 h, the cultures were treated as indicated for 24 h. IFN was used at 240 IU/ml. Some of the cultures were then infected with 1 ml of mengovirus at 1×10^6 plaque-forming units/ml. After additional 24 h, the medium was removed, the cultures were washed over with PBS followed by fixation with methanol and staining with 1:5 dilution of Giemsa stain. Uninfected cells of all clones tested showed the same pattern of staining as shown in Fig. 8 for clone Neo cells (no virus).

Acknowledgments

We thank Dr. Judith Chebath (Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel) for the generous gift of the antifragment B antibodies.

References

Inhibition of 2-5A Synthetase Expression by Antisense RNA

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