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Transcriptional stimulation by $CaPO_4$–DNA precipitates

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ABSTRACT

Genes in human chromosomes that normally require induction by α-interferon are activated after calcium phospate ($CaPO_4$) transfection, but not after DEAE-dextran transfection. The c-fos gene and genes stimulated by γ-interferon also are affected by $CaPO_4$-DNA precipitates, but the calcium ionophore A23187 stimulates only c-fos among this group. These results suggest caution not only in choosing gene transfer methods, but also in interpreting experiments aimed at understanding the role of second messengers in gene activation.

INTRODUCTION

Introducing genes contained as segments within cloned bacterial plasmids into recipient cells followed by monitoring expression of the newly introduced gene is now one of the most common techniques in molecular biology. Infrequently, however, is the impact of the method of introduction on the subsequent function of the gene considered. We have encountered a situation involving major differences in expression of chromosomal human genes normally stimulated by interferon (IFN) when cells have been exposed to $CaPO_4$-DNA precipitates compared to DEAE dextran plus DNA.

Treatment of cultured fibroblasts or HeLa cells with type I interferon (α or β) causes a stimulation of synthesis of a dozen or more proteins that were not formed or formed at low rates before IFN treatment (1-5). Several cDNA clones complementary to mRNAs encoding these proteins have been selected and characterized (1,6-11). Among these are ISG15, ISG54, and ISG56, signifying *i*nterferon *s*timulated *g*enes encoding 15, 54 or 56 kD proteins. It has been shown for each of these genes that stimulation of transcriptional initiation is the basis for this IFN-dependent gene regulation (6,8,12). We have also isolated genomic clones complementary to two of these cDNAs (ISG54 and ISG15) and examined the ability of sequences in the vicinity of the RNA start site to receive and mediate the transcriptional stimulation brought about by IFN-α (12,13).

Originally we had intended to localize this "interferon-stimulated response element" by introducing plasmids containing various upstream regions of an IFN sensitive gene into cells by the widely used $CaPO_4$ technique (14). Several experiments of this type proved unsuccessful

because of high levels of expression from the plasmid in the absence of IFN. Similarly, constitutive expression and an apparent lack of IFN-inducibility in transient assays after $CaPO_4$-DNA transfections have been reported for the mouse interferon-stimulated 202 gene (15,16). In contrast, introduction of DNA by the DEAE dextran method does not obscure the IFN-inducibility of either the endogenous or newly introduced gene (13). After considerable consternation and effort we discovered what we believe to be the basis of the difficulty in these experiments. Independent of interferon treatment, the treatment of cells by the $CaPO_4$-DNA precipitate technique induces both endogenous gene activity and activity of recombinant genes. These findings might or might not eventually prove useful in implicating fluctuating intracellular Ca^{++} levels in control of genes by interferon, but we felt the experiments were of sufficient practical value to warrant description now.

MATERIALS AND METHODS

Cell Cultures and Treatments

HeLa cell clone S_3, ATCC CCl2.2, and FS2 diploid fibroblasts (provided by E. Knight) were cultured in Dulbeccos's Modified Eagle's Medium plus 10% fetal bovine serum and passaged by trypsinization. $CaPO_4$-DNA precipitates were prepared and applied as described (14). Interferon-α_A (Hoffmann LaRoche) was prepared at 10^6 units/ml in media. Calcium ionophore A23187 (Sigma) was dissolved at 10 mg/ml in dimethylsulfoxide. After being cultured and treated as described for each figure, cell monolayers were scraped and washed with phosphate buffered saline, then used to prepare mRNA by NP40 lysis, phenol extraction and ethanol precipitation (13); or for nuclear run-on assays (8).

Probes and Hybridization

Labeled anti-sense RNA probes were used to assay for mRNA content by nuclease protection (17). Nascent RNA labeled in isolated nuclei was extracted and hybridized to 2 μg of each DNA probe applied to nitrocellulose filters using a slot-blot manifold (BRL). Each panel of probes was hybridized with 3×10^6 CPM of labeled run-on transcripts. The specific probes used are as follows: chicken β-actin (18) is a Pst I fragment of cDNA subcloned into pGem1 (Promega Biotec) by N.R., mouse β-globin is genomic fragment D, which spans the polyadenylation site (19); ISG15 is a pGem1 subclone of a 3' Pst I genomic fragment which includes approximately 550 base pairs upstream from the ISG15 polyadenylation site (12); ISG54 is a 560 base pair exon 2 genomic Eco RI fragment in pGem1 and ISG56 is a 3' cDNA subclone of 400 base pairs in pGem2 (D.L., unpublished); pIFNγ-31 and -9 are cDNA clones in pUC12 (9, A.D. Luster, PhD thesis, Rockefeller University, 1987); human β-interferon is a full length cDNA clone (provided by E. Knight); human c-fos is genomic subclone pF4 (provided by R. Prywes), which spans the initiation site (20); human c-myc is genomic subclone pKW20 (21, provided by W.S. Hayward), which contains upstream and first exon sequences from -608 to +210; human hsp70 is cDNA clone pHSG (22); and pGem 1 was used as a negative control for specificity of hybridization.

RESULTS AND DISCUSSION

$CaPO_4$-DNA precipitates cause accumulation of ISG mRNA

HeLa cells were harvested either untreated, 2h after addition of 500 U/ml IFN-α, or 8h after addition of 1 μg/ml salmon sperm DNA as $CaPO_4$-DNA precipitate, then cytoplasmic mRNA was assayed. Figure 1 is typical of results that show treatment with either $CaPO_4$- DNA precipitates or IFN causes equal increases in the level of mRNA transcribed from the endogenous ISG54, -15, and -56 genes. In contrast, the actin mRNA level is the same in control and treated cells. FS-2 diploid fibroblasts were affected in the same manner (data not shown). Accumulated mRNA can be detected for at least 24 to 48 h after the original $CaPO_4$ DNA precipitate treatment. The probes used to assay for the presence of RNA complementary to the interferon-stimulated genes detect the normal 5' RNA initiation sites. Thus the increase in mRNA after addition of IFN or treatment with $CaPO_4$-DNA precipitates is of molecules that initiate at the correct start site. HeLa cells transfected with chimeric constructs which included the ISG54 promoter also accumulated specifically initiated mRNA at the same high levels whether or not the cells received IFN (D.L., unpublished).

Treatment of the cells with the same transfection solutions without DNA *does not* cause the increase in these mRNA's (data not shown). Thus the calcium ions bound to DNA might be responsible for this effect.

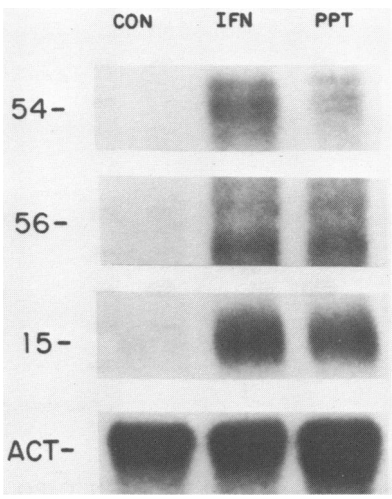

Figure 1. Cytoplasmic mRNA accumulation. Total cytoplasmic RNA was extracted from control cells (CON), 2h after treatment with 500 U/ml of IFNα (IFN), or 8h after treatment with $CaPO_4$-DNA precipitate (PPT). The precipitate was made with salmon sperm DNA and the cells received 1 μg/ml of DNA in the media. The RNA from each sample was assayed by nuclease protection with probes that detect the correct initiation site of either ISG54, ISG56, or ISG15. A probe for actin mRNA was used to demonstrate that equivalent amounts of RNA from each sample were assayed.

DEAE transfection of the same DNA plasmid constructions, while perhaps not quite so effective in delivering DNA into cells, did not stimulate the activity of endogenous genes, and under these circumstances IFN-induced transcription from the transfected plasmids could be observed (13). Alternatively, in most but not all experiments, interferon induction could be measured late (72h to 96h) after $CaPO_4$-DNA precipitate transfection (12).

Transcriptional basis for induction by $CaPO_4$-DNA precipitates.

We wished to determine whether the increase in the IFN-stimulated gene expression after $CaPO_4$-DNA precipitate treatment was based on increased transcription. We tested the relative transcription of cellular genes not affected by any of the treatments employed (actin, globin) and three genes (ISG15, -54, -56) that are affected by IFN-α (6,8,12,13). In addition we examined several other human genes (γ IFN inducible genes, β-interferon, c-fos, c-myc, and hsp70), whose transcriptional regulation under different conditions has been studied (5,9,16,20-39; these

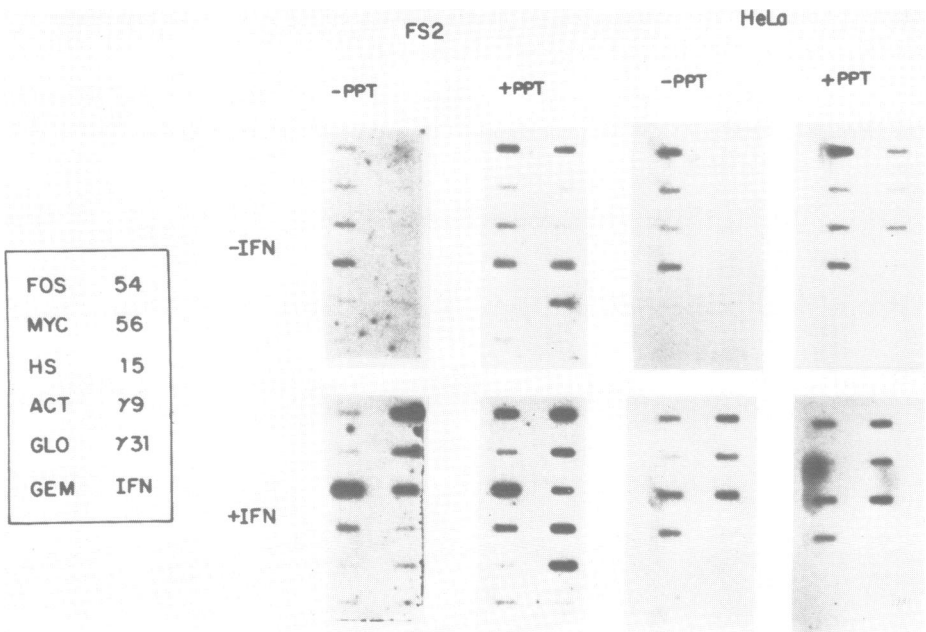

Figure 2. Transcription in cells treated with either IFN, $CaPO_4$-DNA precipitate, or both. Nuclear run-on assays were performed with 1-3 x 10^7 nuclei isolated from untreated cells, cells treated for 1h with 500 U/ml of IFNα, cells 5h after addition of $CaPO_4$-DNA precipitate, or cells treated in the same way, but with 500 u/ml of IFNα added in the last hour before harvest. $CaPO_4$-DNA precipitates were made with M13mp18 RF DNA and the cells received 1 μg/ml of DNA in the media. The experiment was performed with both FS2 and HeLa cells, as shown. Probes for the human genes ISG54, -56, -15, γ-IFN-inducible clones 9 and 31, β-interferon, c-fos, c-myc, and hsp70; chicken β-actin; mouse β-globin; and pGem1 control were bound to nitrocellulose filters in the pattern shown in the key.

citations are representative of a much larger body of work). For each experiment, the actin control signal was similar for all samples, thus allowing a direct comparison between samples in an experiment. Transcription from the β-globin gene, which should be stably repressed in non-erythroid cells, was not detected under any of the conditions used for these experiments. This suggests that the effects observed were limited to genes poised for regulated transcriptional responses. Any hybridization to the pGem probe may be due to the homologous polylinker in the M13 DNA used in transfections.

The transcription of each of these genes was tested with and without IFN stimulation and in the presence or absence of $CaPO_4$- DNA precipitates in both FS2 and HeLa cells (Figure 2). It has been found in a variety of cell lines that ISG15, -54, and -56 reach maximal transcription rates at about 1 h after treatment with IFN (6,8,12,13 and R.P., unpublished observations). Thus, as expected, IFN-α greatly stimulated the transcription of ISGl5, -54 and -56. However, without IFN the $CaPO_4$ precipitate also stimulated all three genes. Although the stimulation was only about 20% of that found after IFN treatment, it was detectable 1 h after addition of precipitate to cells (data not shown) and lasted for at least 5h. Furthermore, densitometric analysis (not shown) revealed that treatment of the ISGs with IFN plus $CaPO_4$-DNA precipitate does not yield an additive effect.

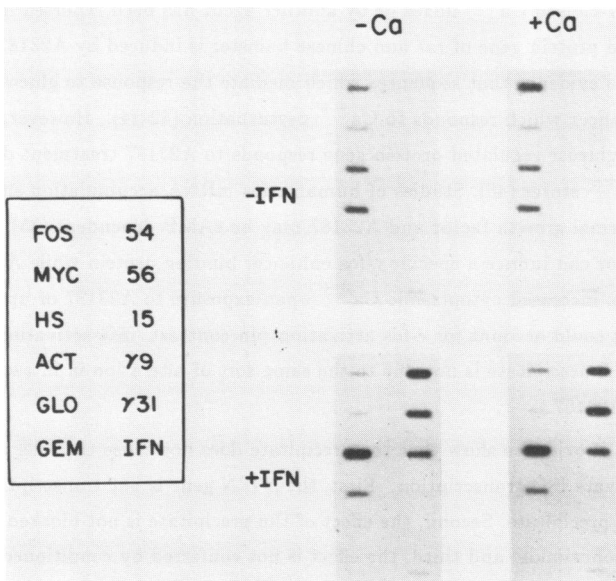

Figure 3. Transcription in cells treated with either IFN, A23187 or both. Nuclear run-on assays were performed with nuclei isolated from 3 x 10^7 HeLa cells for each of the following samples: untreated, treated with 500 U/ml of IFNα for 1h, treated with 10 μM A23187 for 1hr, or treated for 1h with both agents simultaneously. Probes are as described in Fig. 2 legend and arranged in the pattern shown in the key.

All possibly highly regulated or "volatile" genes are not handled in the same manner: the γ-IFN inducible genes did not respond to the α-IFN but were induced by the precipitate; β-interferon and c-myc transcription were unchanged by either treatment; the transcription of c-fos was not affected by IFN but was stimulated by the precipitate; while the heat shock gene was not stimulated by $CaPO_4$ DNA precipitate but was transcriptionally induced by IFN treatment. This latter observation is a novel finding which warrants further study in its own right.

Separate effects of free Ca^{++} and $CaPO_4$-DNA precipitate.

It was previously shown that the transcriptional response of the ISG54 and ISG56 genes to IFNβ did not occur in diploid fibroblasts treated with the Ca^{++} ionophore A23187 (8), which equilibrates calcium ions across biological membranes (40). The concentration of the ionophore used in those experiments was known to be sufficient to speed mouse erythroleukemia cell committment to induced differentiation (41). This treatment was retested with a higher dose of A23187 applied to HeLa cells, and transcriptional stimulation of the c-fos gene served as a positive control (20). Again, ISG54 and -56 did not respond to the effect of the ionophore, and neither did ISG15 or any of the other genes newly tested, except c-fos (Figure 3).

$CaPO_4$-DNA precipitates may very likely cause transcriptional activation of different genes by different mechanisms. Consistent with this conclusion, transcriptional induction of a gene either by A23187-caused Ca^{++} fluxes or by another agent has been reported in other cases. A glucose-regulated protein gene of rat and chinese hamster is induced by A23187 treatment as well, and there is evidence that sequences which mediate the response to glucose starvation overlap the enhancer which responds to Ca^{++} perturbation (42-44). However, it has been shown that the glucose-regulated protein gene responds to A23187 treatment due to depletion of intracellular Ca^{++} stores (45). Studies of human c-fos mRNA accumulation show that effects from both epidermal growth factor and A23187 may be cAMP dependent (35), but that epidermal growth factor can induce a specific c-fos enhancer binding protein while A23187 does not (20). In this case, increased cytoplasmic Ca^{++} upon exposure to A23187 or uptake of $CaPO_4$-DNA precipitate could account for c-fos activation. In contrast, ISG activation by treatment with $CaPO_4$-DNA precipitate is not due to the same sort of alteration in intracellular Ca^{++} as that caused by A23187.

Three lines of evidence show that the precipitate does not cause the cells to secrete IFN and thereby activate ISG transcription. First, the β-IFN gene is not transcriptionally induced by $CaPO_4$-DNA precipitate. Second, the effect of the precipitate is not blocked by pretreatment of cells with cycloheximide, and third, the effect is not conferred by conditioned medium from cells treated with precipitate (D.L., unpublished observations).

In practical terms, we have been able to avoid a calcium shock to the transcriptional apparatus either by introducing the recombinant DNA into cells by the DEAE method or by inserting the IFN-sensitive promoter into adenovirus vectors which can enter cells in normal

medium (46). However, this report reinforces the need to exercise caution in suggesting a physiological role for second messenger pathways wherever some "general" effector such as high levels of cAMP, calcium ions or phorbol esters, which may radically affect cultured cells, are the agents of stimulated genetic activity.

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