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Identification of interferon-modulated proliferation-related cDNA sequences

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ABSTRACT **To identify genes mediating the antiproliferative action of interferon (IFN), two cDNA libraries were constructed with mRNA from IFN-treated and untreated human fibrosarcoma (HT1080) cells previously shown to be highly sensitive to the antiproliferative effects of IFN. Differential screening of these two libraries identified cloned sequences whose expression was either induced or repressed with IFN treatment. Rescreening of these sequences with cDNA probes constructed from proliferating or quiescent cells led to the identification of one IFN-induced and three IFN-repressed sequences whose expressions also appeared to be modulated by cell proliferation. Blot-hybridization analysis revealed that RNA levels corresponding to the three repressed genes decreased when HT1080 cells were treated with IFN or when proliferation of normal CUA foreskin fibroblast cells became naturally arrested by contact inhibition. Levels of RNA corresponding to the induced gene increased in HT1080 cells within 24 hr after IFN-treatment but declined below basal levels by 48 hr. Expression of these genes was unaffected or only slightly affected by IFN treatment in variant cells resistant to the antiproliferative effects of IFN. Collectively, these results suggest that the identified cDNAs correspond to genes that are involved in the antiproliferative action of IFN. Moreover, these results also suggest that IFN's antiproliferative action may be exerted through genes that contribute to arresting cell proliferation during contact inhibition.**

Regulation of cell proliferation is likely to be a complex process involving the coordinate expression of discrete genes. Consistent with this notion is the observation that transcriptional activity is required for cells to initiate proliferation from a resting state (1, 2). Attempts to associate specific changes in gene expression with cell proliferation have resulted in the identification of several uncharacterized genes from yeast (3), rodent (4), and human cells (5). Known genes, such as the oncogenes c-*myc* (6, 7), c-*fos* (8, 9), and c-*ras* (7, 10), the gene encoding cellular tumor antigen p53 (11), and other genes coding for well-characterized proteins such as ornithine decarboxylase (12), β-actin (7, 13), thymidine kinase (14), and histones (15, 16) also have been shown to be differentially expressed during proliferation. However, the causal relationships between the expression of these genes and the regulation of cell proliferation remain obscure. Some of these genes appear to be modulated only as a consequence of proliferation and do not directly contribute to proliferation controls.

Approaches used in these studies have emphasized the initial events involved in the commitment of a quiescent cell to undergo DNA synthesis or in the transition from the G_1 to S phase of cycling cells. An alternative perspective of cell proliferation controls is one that focuses on the regulatory elements that arrest the proliferation of dividing cells and restore them to the quiescent state. An approach consistent with this rationale is the use of growth antagonists in arresting or regulating cell proliferation. Interferon (IFN), a biological response modifier, is one such growth antagonist capable of inhibiting cell proliferation (17). Recently, we have shown that IFN at low doses is capable of inducing contact- and shape-sensitive controls in malignant human fibrosarcoma (HT1080) cells (18). These controls, which are not commonly observed in malignant cells, result in the arrest of cell proliferation when normal cells *in vitro* come into contact with one another or when their shapes are distorted to a more rounded form (18–20). The ability of IFN to confer to unregulated tumor cells a level of proliferation control characteristic of normal fibroblasts suggests a possible relationship between gene expression mediated by IFN and those genes expressed in the maintenance of regulated cell proliferation. Guided by this premise, we examined IFN-modulated gene expression in IFN-treated and untreated HT1080 cells and the correlation of this expression to cell proliferation.

MATERIALS AND METHODS

Culture Conditions and IFN Treatment. Stock cultures of HT1080 (21) and CUA cells were grown in Eagle's minimal essential medium (EMEM) supplemented with either 5% (HT1080 cells) or 10% (CUA cells) fetal bovine serum (FBS). CUA cells were used only at passage numbers of <20 population doublings. Subconfluent cultures of HT1080 cells were treated with highly purified human fibroblast IFN (1×10^6 to 1×10^7 international research units per mg of protein; HEM Research, Rockville, MD and Lee BioMolecular Research Laboratories, San Diego, CA) at a concentration of 120 international research units per ml for various times prior to the isolation of mRNA.

RNA Isolation and Library Construction. Total RNA was extracted by cell lysis in a buffer of 6 M guanidinium hydrochloride by the method of Chirgwin *et al.* (22) and purified by CsCl centrifugation (22, 23). Cytoplasmic RNA was prepared by a modified Favalaro procedure (24). IFN and control cDNA libraries were constructed using poly(A)-selected mRNA (25) isolated from IFN-treated and untreated HT1080 cells. Double-stranded cDNA was synthesized from this RNA by a variation of the Gubler and Hoffman (26) procedure and was inserted by G·C-tailing into the *Pst* I site of pBR322. Annealed vector cDNA was then used to transform competent *Escherichia coli* HB101 cells by the method of Hanahan (27).

Differential Screening of the cDNA Libraries. Tetracycline-resistant ampicillin-sensitive colonies were individually transferred to 96-well microtiter plates and replica-plated onto duplicate Whatman 541 paper filters. The filters were

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Abbreviation: IFN, interferon.
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incubated on chloramphenicol plates for plasmid amplification and then processed for hybridization as described by Gergen *et al.* (28). cDNA sequences corresponding to genes whose expressions were induced or repressed by IFN treatment were identified by screening the duplicate filters from the IFN and control cDNA libraries with [^{32}P]cDNA probes complementary to poly(A)-RNA from IFN-treated (120 international research units per ml, 18 hr) and untreated HT1080 cells. Clones representing IFN-induced and IFN-repressed sequences were selected and screened with [^{32}P]-cDNA probes complementary to poly(A)-RNA from proliferating and from quiescent contact-inhibited CUA cells. This differential screening identified proliferation-associated cDNAs.

Blot-Hybridization and Slot-Blot Analysis of RNA. For blot-hybridization analysis, RNA was electrophoresed in formaldehyde agarose gels (23) and then transferred to Nytran nylon membranes. Membranes were baked for 2 hr at 80°C and then prehybridized for 24 hr at 37°C in buffer consisting of 50% (vol/vol) formamide, 5× SSPE (0.75 M NaCl/0.04 M NaH_2PO_4/0.004 M EDTA, pH 7.4), 2× Denhardt's solution (0.04% polyvinylpyrrolidone/0.04% Ficoll/0.04% bovine serum albumin), 0.1% $NaDodSO_4$, and 150 μg of sheared salmon sperm DNA per ml. After prehybridization, recombinant plasmid prepared by alkaline lysis (23) and nick-translated to a specific activity of $0.5–2 \times 10^8$ dpm/μg of DNA was added to the prehybridization buffer. After 48 hr, the membranes were subjected to two washes in 0.75 M NaCl/0.075 M sodium citrate, pH 7.0/0.1% $NaDodSO_4$ at room temperature for 15 min, followed by successive 30-min washes at 37°C in 0.15 M NaCl/0.015 M sodium citrate, pH 7/0.5% $NaDodSO_4$ and in 0.015 M NaCl/0.0015 M sodium citrate, pH 7/0.5% $NaDodSO_4$. These filters were then dried and autoradiographed. For slot-blotting, 10 μg of cytoplasmic RNA was serially diluted, applied to Nytran nylon membranes, and then processed as described above for blot-hybridization analysis. Slot-blot results were densiometrically scanned to determine the dilution corresponding to half-maximal hybridization. Relative abundance was estimated by normalizing the reciprocal of the half-maximal hybridization dilution to that obtained with the pREP-2 probe.

RESULTS

Construction of IFN and Control cDNA Libraries. An IFN cDNA library was constructed by using poly(A)-RNA isolated from subconfluent HT1080 cells treated for 17 hr with 400 international research units per ml of IFN-β. This concentration of IFN is sufficient to completely inhibit proliferation of HT1080 cells without cytotoxicity if sufficient time is allowed after treatment. However, the antiproliferative effects of IFN are not fully manifested in these cells until 24 hr after exposure to IFN (29, 30). Since antiproliferative signals triggered by IFN are most likely found in proliferating cells just prior to proliferation arrest, the interval of 17 hr was chosen to ensure the full expression of the later molecular changes associated with IFN's antiproliferative effect without significant decay of the earlier events. A second control cDNA library was constructed using poly(A)-RNA from

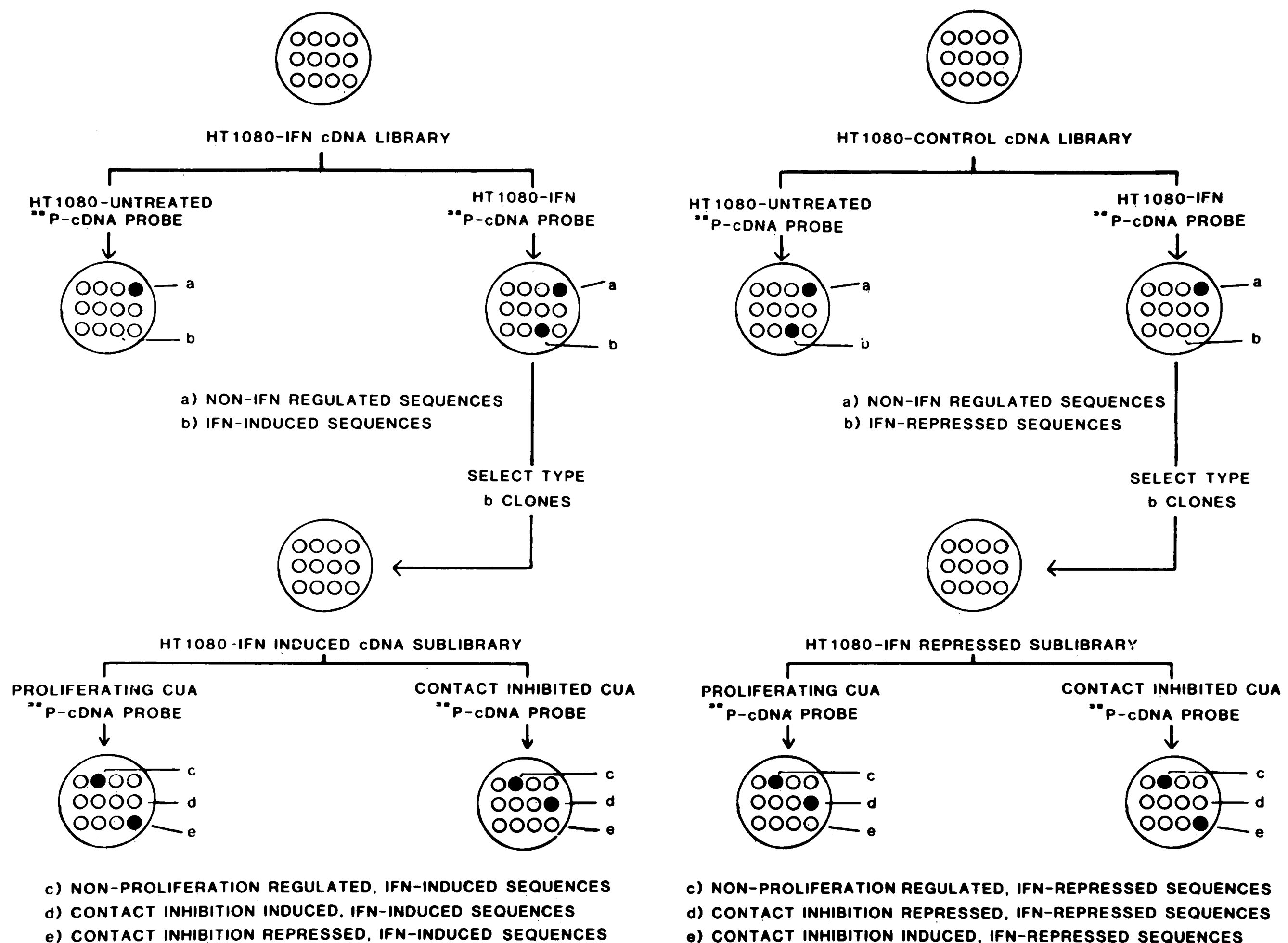

FIG. 1. Differential screening strategy to identify recombinant clones. (*Left*) Screening for IFN-induced contact-inhibition-induced cDNA clones. (*Right*) Screening for IFN-repressed contact-inhibition-repressed cDNA clones.

untreated subconfluent HT1080 cells. Both libraries consisted of >30,000 clones, ≈10% of which were examined in initial screenings.

Screening for IFN-Modulated and Proliferation-Related Clones. Fig. 1 shows the differential hybridization strategy used in the screening of cDNA clones. The IFN and control cDNA libraries were first screened for IFN-modulated cDNAs by separately hybridizing cDNA probes synthesized from IFN-treated HT1080 mRNA (IFN^+ probe) and untreated HT1080 mRNA (IFN^- probe) to duplicate filters containing replica-plated colonies. Clones representing IFN-induced sequences were characterized by hybridization to the IFN^+ probe but not to the IFN^- probe (clone b in Fig. 1 *Left*). Since variations in the efficiency of colony transfer to the filters occasionally result in nonreproducible hybridizations, clones that were identified as being IFN-induced were twice rescreened. Of >2000 IFN cDNA clones screened in this manner, 29 were classified as putative IFN-induced clones. Results of rescreening the IFN-induced sublibrary are shown in Fig. 2 *Upper*. Clones containing IFN-repressed sequences are those from the control cDNA library, which gave positive hybridization with the IFN^- probe but not with the IFN^+ probe (clone b in Fig. 1 *Right*). Of >3,000 control cDNA clones screened, 30 were judged to be IFN-repressed after three rounds of screening (see Fig. 3 *a*, *b*, *e*, and *f*).

To determine if the IFN-modulated cDNAs corresponded to genes associated with the proliferative state of the cell, the IFN-induced and IFN-repressed sublibraries were rescreened with cDNA probes made from the poly(A)-RNA of proliferating (PRO^+ probe) and quiescent (PRO^- probe) CUA cells. Since CUA cells exhibit stringent contact inhibition of proliferation at confluence (18), the RNA for synthesis of the PRO^- probe was extracted from confluent CUA cells. Those clones exhibiting differential hybridization to the proliferation probes were judged to be proliferation-correlated (clones d and e in Fig. 1 *Left* and *Right*). This screening strategy identified 1 of the 29 IFN-induced sequences (pIND-12) as also being induced when cells became contact-inhibited (Fig. 2 *Lower*). Three of the 30 IFN-repressed sequences, pREP-2, pREP-3, and pREP-28, were identified as also being repressed when cells became contact-inhibited (Fig. 3 *c*, *d*, *g*, and *h*).

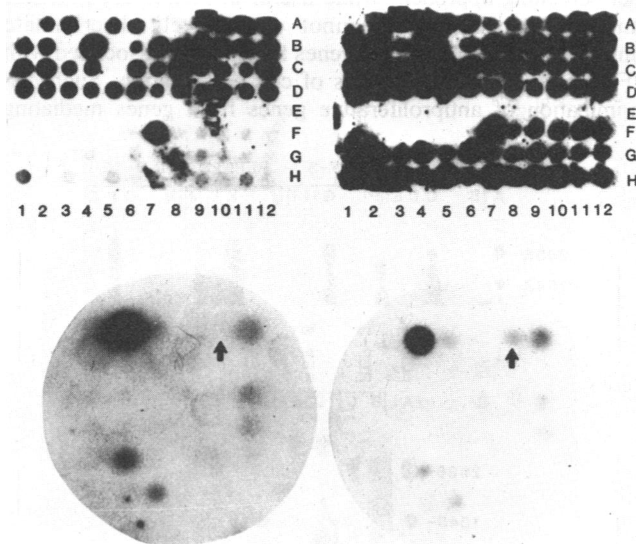

FIG. 2. Differential screening of the IFN-induced cDNA sublibrary. (*Upper Left*) Screening with IFN^- probe. (*Upper Right*) Screening with IFN^+ probe. Clones in rows A–D are nonmodulated clones. Clone at position F7 is a constitutively expressed control. All other clones were identified as being IFN-induced in a previous screening. The pIND-12 clone is located at position H12. (*Bottom Left*) Screening of IFN-induced clones with Pro^+ probe. (*Bottom Right*) Screening of duplicate filter with Pro^- probe. The position of pIND-12 in the bottom filters is indicated by the arrows.

Agarose gel electrophoresis of *Pst* I-digested plasmids confirmed that each contained inserts that varied in size from 190 to 300 base pairs (bp). RNA blot analysis using the plasmids as probes revealed hybridization to different mRNAs, suggesting that the cDNAs of the plasmids were unique. The cDNA insert sizes and the corresponding mRNA sizes are given in Table 1.

Modulation of pREP-2, pREP-3, pREP-28, and pIND-12 Gene Expression by IFN. Selected plasmids were labeled by nick-translation with ^{32}P-labeled nucleotides and were used as hybridization probes for the blot-hybridization analysis of RNAs from IFN-treated and untreated HT1080 cells. All three IFN-repressed plasmids hybridized less to RNA from 18-hr IFN-treated cells than to RNA from untreated cells with further decrease in the degree of hybridization as the duration of the IFN treatment increased to 48 hr (Fig. 4 *Upper*). These results indicate a decrease in the levels of corresponding mRNA following IFN treatment. However, there was a dual response with the pIND-12-associated mRNA, where the levels displayed significant increase by 18 hr after IFN treatment and then diminished by 48 hr post-treatment when compared to the basal levels of this mRNA in untreated cells (Fig. 4 *Upper*, lanes o, p, and q). This result, though unexpected, is consistent with other characterizations of IFN-induced sequences. These other studies showed that the levels of a large number of mRNA sequences are increased within 8 hr after exposure to IFN, but some of these decrease to below the basal levels by 24–48 hr (31, 32). Failure of the probes to hybridize to discrete bands of RNA in samples of cellular RNA depleted of the poly(A)-RNA fraction (Fig. 4 *Upper*, lanes B, F, J, and N) indicated that the observed signals did not result from nonspecific hybridization to ribosomal RNA.

Lack of Modulation by IFN of pREP-2, pREP-3, pREP-28, and pIND-12 Gene Expression in Cells Resistant to the Antiproliferative Action of IFN. The relationship between the above IFN-modulated proliferation-related genes and the antiproliferative action of IFN was investigated in HT1080-IFN^r cells, which are resistant to this action of IFN (29). While being insensitive to the antiproliferative action of IFN, these cells retained their sensitivity to the other actions of IFN such as the establishment of the antiviral state. Nick-translated ^{32}P-labeled plasmids were used to screen blots of RNA extracted from untreated and IFN-treated HT1080-IFN^r cells. Hybridization results (Fig. 4 *Lower*) indicate that the levels of mRNA corresponding to the plasmids pREP-2 and pREP-3 were unaffected by IFN treatment. Levels of mRNA for probes pREP-28 and pIND-12 were affected but to a lesser extent than for their parental HT1080-sensitive cells. These observations are consistent with the genes corresponding to these plasmids participating in the mediation of the antiproliferative effect of IFN.

Modulation of pREP-2, pREP-3, pREP-28, and pIND-12 Gene Expression by Contact Inhibition. The ability of IFN to induce contact- and shape-dependent sensitive controls in HT1080 cells (18) suggested that genes modulated by IFN may also be modulated by contact inhibition of cell proliferation. To evaluate this possibility, the IFN-modulated cDNA plasmids were used to compare blots of RNA from CUA fibroblasts in the actively proliferating logarithmic phase with that from CUA cells after maintenance at confluency for 48 hr. Complete cessation of proliferation occurred for CUA cells kept confluent for this period of time. Results shown in Fig. 5 *Upper* indicate that levels of RNA corresponding to all four cDNA clones declined when cells became contact-inhibited. Quantitation of relative abundance for the RNAs determined from densiometric analysis of slot blots corrob-

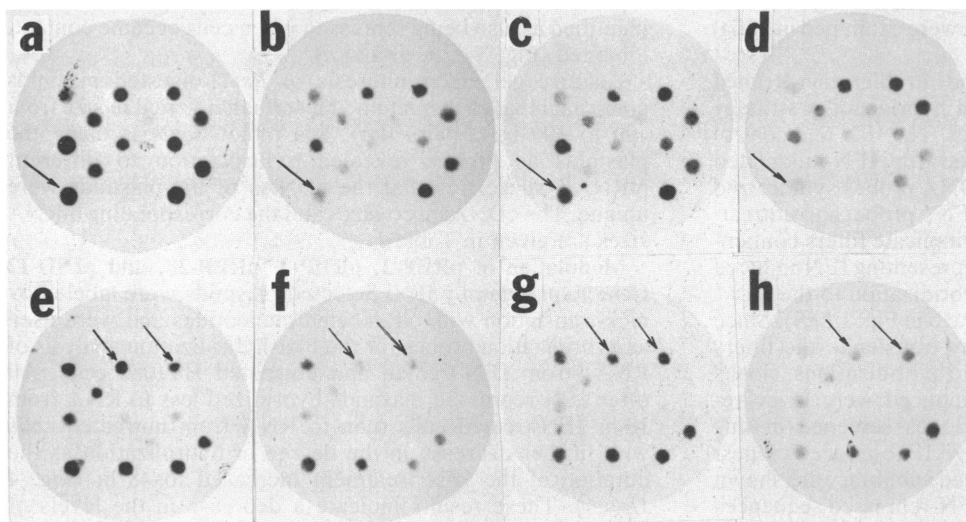

FIG. 3. Differential screening of the IFN-repressed cDNA sublibrary. (*Upper*) One set (*a–d*) of replica filters. (*Lower*) A different set (*e–h*) of replica filters. Filters a and e were screened with the IFN^- probe, b and f with the IFN^+ probe, c and g with the pro^+ probe, and d and h with the pro^- probe.

orated the qualitative results from blot-hybridization analysis (Fig. 5 *Lower*). These changes in mRNA levels are unlikely to be the result of IFN, whose synthesis was induced during contact inhibition, since the continuous presence of 100 neutralizing units per ml of monospecific antibody to human fibroblast IFN had no influence on the changes in the mRNA levels observed during contact inhibition. The similar repression of pREP-2, pREP-3, and pREP-28 gene expression resulting from IFN treatment or from contact inhibition is suggestive of these genes playing a basic role in inhibiting cell proliferation.

DISCUSSION

The ability of IFN to profoundly influence cell proliferation controls suggests that included among the genes whose expressions are affected by IFN are those that participate in regulating cell proliferation. On this basis, cDNAs corresponding to genes induced or repressed with IFN treatment were screened with proliferation-related probes. This approach led to the identification of sequences whose expressions are modulated by exposure to IFN and are similarly modulated when cell proliferation becomes "naturally" arrested by contact inhibition. By virtue of the fact that these sequences satisfy two independent screening criteria (i.e., modulation by IFN *and* modulation in parallel manner by contact-inhibition), it is likely that they are related to the antiproliferative effect of IFN. Nonrelatedness to IFN's antiproliferative effect would require the fortuitous coincidence of differential screening to fulfill the two criteria—a possible but unlikely prospect.

Direct examination of mRNA levels corresponding to the repressed genes confirmed that expression of these genes was suppressed in HT1080 cells upon treatment with IFN and suppressed in CUA cells when proliferation became contact-inhibited. In the case of mRNA levels corresponding to the induced gene, induction was observed in HT1080 cells only within 24 hr after IFN treatment, after which levels of mRNA decreased. The persistence of enhanced mRNA levels for such a short period indicates that, if this gene is involved in mediating the antiproliferative effect of IFN, its continued expression is not necessary for the maintenance of the antiproliferative effect. Elevation of mRNA levels corresponding to the induced gene was not observed in CUA cells 48 hr after reaching confluency. Failure to observe enhancement after contact inhibition may reflect the transient nature of pIND-12 induction, where mRNA levels for this gene increase during the early stages of contact inhibition but decline by the time the cultures become fully confluent.

IFN is known to exert a diversity of effects on cells in addition to its antiproliferative effect. Consequently, the majority of genes modulated by IFN are probably not involved in mediating the antiproliferative effect. The screening of IFN-modulated cDNAs with proliferation-related probes was undertaken to distinguish the antiproliferative genes. However, this differential screening approach, while useful in identifying potential antiproliferative genes, cannot conclusively discriminate antiproliferative genes from genes fortuitously associated with IFN-treatment and/or phases of cell proliferation. Such discrimination of antiproliferative genes from genes mediating

Table 1. Characterization of IFN-modulated, proliferation-related recombinant plasmids

Plasmid	Insert size, bp	Corresponding mRNA species, bp
pREP-2	195	2020
pREP-3	225	2250
pREP-28	210	2375, 1500
pIND-12	290	2100

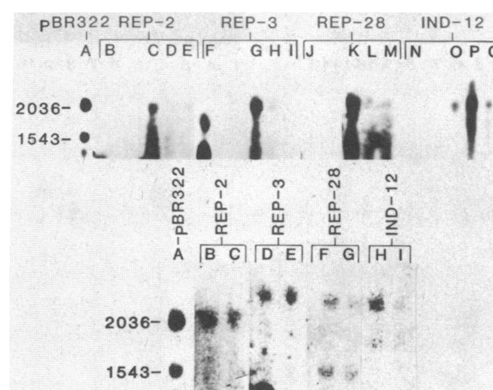

FIG. 4. (*Upper*) Blot-hybridization analysis of HT1080 RNA extracted from untreated cells (lanes C, G, K, and O) and from cells treated with IFN for 18 hr (lanes D, H, L, and P) and from cells treated with IFN for 48 hr (lanes E, I, M, and Q). Lanes B, F, J, and N contain cellular RNA depleted of the poly(A) fraction. (*Lower*) Blot-hybridization analysis of RNA extracted from HT1080-IFN^r variant untreated cells (lanes B, D, F, and H) and from cells treated with IFN for 48 hr (lanes C, E, G, and I). The identities of the nick-translated plasmids used as probes are shown directly above the lanes.

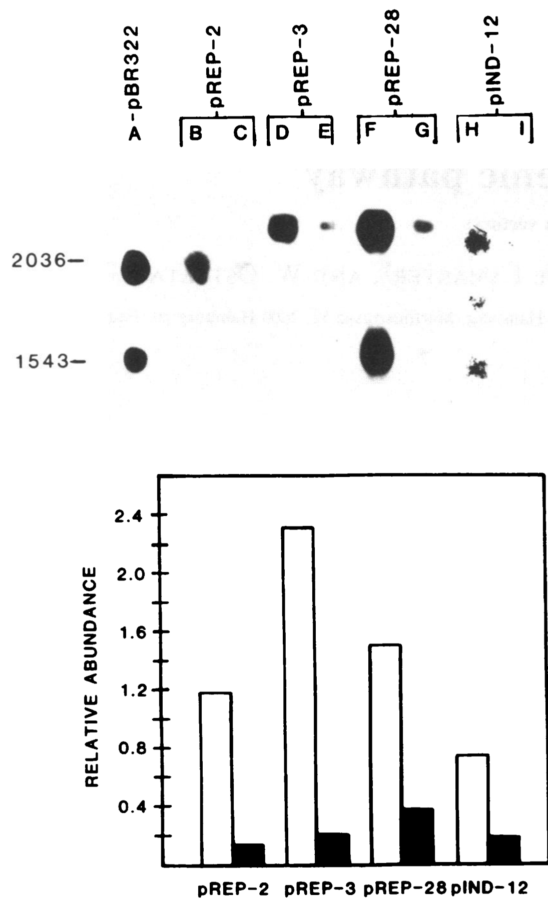

FIG. 5. (*Upper*) Blot-hybridization analysis of RNA from proliferating and contact-inhibited CUA cells. Lanes: A, pBR322 fragments used as size markers; B, D, F, and H, RNA isolated from proliferating cells; C, E, G, and I, RNA isolated from contact-inhibited cells. (*Lower*) Relative abundances of RNA corresponding to pREP-2, pREP-3, pREP-28, and pIND-12 cDNA sequences determined from densiometric scanning of slot blots. □, Levels of RNA from proliferating cells; ■, levels of RNA from confluent cells.

other actions of IFN could be facilitated by the analysis of cells resistant to the antiproliferative action of IFN but sensitive to all other actions of IFN. Since the HT1080ʳ variant cells used in our study responded to IFN by establishing an antiviral state, they are presumably defective only in the elements contributing to the antiproliferative effect. The observation that expression of pREP-2 and pREP-3 corresponding genes were unaffected by IFN-treatment in HT1080ʳ cells lends credence to their involvement in the antiproliferative action. The minimal modulation in HT1080ʳ resistant cells of pREP-28 and pIND-12 genes does not preclude their participation in the antiproliferative effect since their modulation was generally less than for sensitive HT1080 cells.

IFN is capable of exerting two types of antiproliferative effects. One type, evident at higher concentrations, results in inhibiting the proliferation of subconfluent cells (17). The second type, evident at lower concentrations, results in inducing shape- and contact-sensitive proliferation controls in malignant cells that originally lacked them (18). Characterization of HT1080ʳ cells in our laboratory has established that these cells are capable of proliferation in growth-inhibitory concentrations of IFN under subconfluent conditions, but proliferation becomes arrested when the cells become confluent, regardless of whether IFN is present or not (18). Apparently, HT1080ʳ cells are defective in responding to the first type of antiproliferative effect but not necessarily to the second type. In this light, the occurrence of some modulation of pREP-28 and pIND-12 genes suggests that they may be more involved in controls related to contact inhibition.

The congruence of three independent expression characteristics—namely, (*i*) modulation by treatment with IFN; (*ii*) similar pattern of gene modulation as for IFN treatment, which resulted when proliferation was arrested by contact inhibition; and (*iii*) absence of or minimal modulation in HT1080ʳ cells—is strongly indicative that some or all of the genes corresponding to pREP-2, pREP-3, pREP-28, and pIND-12 are involved in the antiproliferative action of IFN. Moreover, these results taken together also suggest that the antiproliferative effect of IFN is exerted through genes which contribute to arresting cell proliferation during contact inhibition. For genes corresponding to the repressed sequences, their expression actually may be required for proliferation, and suppression of expression may lead to arrest of proliferation.

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