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MOLECULAR CLONING, SEQUENCING AND EXPRESSION OF HUMAN INTERFERON-γ-INDUCIBLE INDOLEAMINE 2,3-DIOXYGENASE cDNA[1]

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The antiproliferative action of human interferon (HuIFN)-γ on human cells and the inhibition of intracellular pathogens, e.g. Toxoplasma gondii and Chlamydia psittaci, is at least in part due to an induction of indoleamine 2,3-dioxygenase (IDO) enzyme which degrades tryptophan, an essential amino acid. A cDNA clone (called C42) was isolated from a cDNA library made from poly(A)$^+$ RNA obtained from HuIFN-γ-treated human fibroblasts. Its nucleotide sequence revealed an open reading frame coding for a polypeptide of 403 amino acids, but no homology with any known gene in GenBank database was found. Evidence was obtained indicating that this cDNA codes for IDO: (i) Hybrid selected C42 specific poly(A)$^+$ RNA from IFN-γ-treated human cells coded for a polypeptide in vitro of ~42 kD (reported size of IDO, ~40 kD) which was immunoprecipitated by monoclonal anti-IDO antibody but not by a control antibody; and (ii) transfection of human fibroblasts with an expression plasmid containing C42 cDNA transcribed from chicken β-actin promotor led to constitutive expression of C42 specific RNA as well as IDO activity. This cDNA clone will be useful in studying the role of IDO in the biological effects of IFN-γ, and the regulation of IDO gene by IFN-γ.

Interferon (IFN)-γ is a cytokine produced by T-cells (and large granular lymphocytes) upon immunological or mitogenic stimulation (1). The multiple biological effects of IFN-γ are, at least in part, mediated through changes in cellular gene expression (Ref. 2-8 for reviews). For example, the antiproliferative effect of IFN-γ on a spectrum of tumor cells and the inhibition of intracellular pathogens, such as Toxoplasma gondii and Chlamydia psittaci in host cells is at least partly due to the induction of indoleamine 2,3-dioxygenase enzyme (IDO; EC 1.13.11.17), which catalyzes the degradation of L-tryptophan (an essential amino acid) to N-formylkynurenine (9-13). This enzyme is induced strongly by IFN-γ in a number of cell types but rather poorly by IFN-α or -β (9, 16), and therefore represents an example of cellular genes whose expression is regulated differentially by IFN-γ as against IFN-α and -β. Here we report the isolation of a near full length cDNA clone corresponding to an IFN-γ-inducible mRNA, its nucleotide sequence, and the identification of its protein product as IDO.

[1] Some of this work was presented at the Annual International Conference on Interferons and Cytokines, October 22-27, 1989, Florence, Italy.

Abbreviations: cDNA, complementary DNA; HuIFN, human interferon; IDO, indoleamine 2,3-dioxygenase; kb, kilobase; kD, kilodalton.

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METHODS

Isolation of cDNA Clones and Sequencing. Human diploid fibroblast cells (FS-4) were grown in roller bottles (17), treated with purified recombinant HuIFN-γ (500 units/ml) in the presence of cycloheximide (50 μg/ml) for 18 hr, and total RNA was isolated (18). Poly(A)$^+$ RNA was obtained by two cycles of chromatography on oligo(dT)-cellulose (19) and used as template for the synthesis of double-stranded cDNA essentially as described (20). The cDNA was methylated with EcoR I methylase, blunt ended by incubation with Klenow fragment of DNA polymerase I and deoxynucleotides, and ligated with EcoR I linkers. After digestion with EcoR I, the cDNAs larger than 0.6 kb were selected by agarose gel electrophoresis and electroelution, ligated into the arms of λZAP II vector (Stratagene), packaged in an in vitro packaging extract and used for infecting Escherichia coli XL1-Blue host. Approximately 6 x 10^4 independent plaques were screened by hybridization with a previously isolated partial cDNA clone (called C5-4, ~1 kb) that is complementary to an IFN-γ-inducible mRNA (17). Three positive clones with inserts ranging from 1.3 to 2.0 kb were isolated, and the cDNA inserts from these clones were rescued with helper phage R408 into Bluescript SK(-) phagemid (21). The clone with ~2 kb cDNA insert (called C42) was mapped, and restriction fragments were subcloned into M13mp18 and M13mp19 phages. Single-stranded M13 phage DNAs were isolated and sequenced by the dideoxy chain termination method (22) using sequenase kit (U.S. Biochem. Corp.).

Immunoblot Analysis. FS-4 cells were incubated with or without HuIFN-γ (300 units/ml, 24 hr.) and lysed in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% NP-40 detergent. Cleared lysates (12,000 g, 10 min.) were fractionated on 15% SDS-polyacrylamide gel (23), and the fractionated proteins were transferred onto nitrocellulose membrane (24). The blot was incubated with mouse monoclonal anti-IDO antibody (15, 2 μg/ml) in TBS/T (10 mM Tris-HCl, pH 7.5, 250 mM NaCl) containing 0.15% Tween 20 for 1 hr, rinsed with TBS/T buffer and then incubated with goat anti-mouse IgG conjugated with biotin (BRL). The blot was washed with TBS/T and then incubated with streptavidin conjugated with alkaline phosphatase (BRL). After further washing with TBS, the blot was developed by incubation with BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) and NBT (nitroblue tetrazolium chloride) as recommended by the supplier (BRL).

Other procedures used are described in figure legends.

RESULTS AND DISCUSSION

Isolation of a Near Full Length cDNA Clone for a HuIFN-γ-Regulated mRNA. A cDNA library was prepared in λZAP II vector from poly(A)$^+$ RNA isolated from FS-4 human fibroblasts pretreated with HuIFN-γ, and screened with a previously isolated partial cDNA clone called C5-4 (17). Three new cDNA clones were identified (named CII, CIII and C42) which extended C5-4 cDNA clone by 0.3 kb, 0.7 kb and 1.0 kb, respectively (Fig. 1). Clone C42 with the longest insert (~ 2 kb) was analyzed in more detail. The C42 cDNA hybridized to a 2.2 kb mRNA from HuIFN-γ-induced cells on Northern blot (not shown) as observed earlier with C5-4 cDNA as a probe (17). To determine whether the C42 cDNA had any homology to any known gene, its nucleotide sequence was determined (Fig. 2), which revealed a single open reading frame that encodes a polypeptide of 403 amino acids with a calculated Mr of 45,332. It was noted that the open reading frame was preceded by a long untranslated sequence and that a Kozak consensus sequence, CCA/GCC (25), was not found before the first ATG of the open reading frame. The sequence obtained did not contain a polyadenylation signal at the 3'-end, which was not surprising since the C42 cDNA is shorter at the 3'-end as compared to the C5-4 cDNA by about 150 nucleotides (Fig. 1). A comparison of the C42 cDNA sequence with the GenBank and NBRF database revealed no identity with any known gene sequence at either nucleotide level or amino acid level.

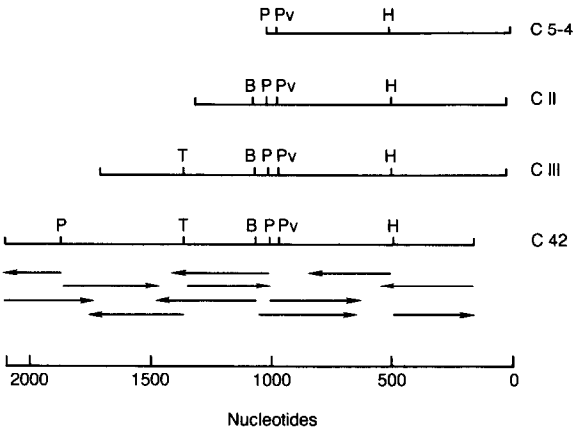

Fig. 1 Restriction map of cDNA clones isolated with C5-4 cDNA (17) as a probe, and strategy used for sequence analysis. B, BamH I; H, Hind III; P, Pst I; Pv, Pvu II; T, Taq I.

Evidence Indicating that C42 cDNA Codes for IDO. It was demonstrated earlier that IFN-γ-inducible C5-4 specific mRNA obtained by hybrid selection coded for a polypeptide of Mr ~42,000, and that IFN-γ treatment induced the transcription of the C5-4 specific cellular gene but it required de novo synthesis of some new protein which

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                                                                                                    TGAGAAGGGCAAATGCTATCATTGGAAAAACTGACAAAAGTCCC    44
AATAGGAAAAATAAGGAAGTGGAGAGTTACTATGTTTCTAATTTTTCATGTGCTTCTATTTTTTTCCTACTTCAGAGCCATTGACTAATAGTTGAGTATAACACAGGTTGTGTTTCCGG  163
GCTGCTGAAACATGACACTAATATTTTCAAAGAACTGTGGAAGCCTAAAAGGAAGCCAATGAGAAATAACTAAATGAGAGTTTAGGACTGCAGCCTTCATTTTCATTCAAAGATTTAAA  282
AGTTTCCATAAAGTAAAATGTTCTTCTCCGGCCACCTGTTTTCATAGTTCTGTGTTTTCCTTCAGGCCTTTCTGGCTTCCTATATGGCAGTAAGAAAATGATGTGCTTAATGATTACAA  401
ATTTCATATGGAATACGAACTTTCAGTTTGTACATATGATGCACAGAGATGCTTTTGTGGTTTTATTGGTTTTCATATTACAAACAAAGAAACTAGAAAATGAAACCATTCCAAAAGTG  520
GAAGTAATTTCTCACTGCCCCTGTGATAAACTGTGGTCACTGGCTGTGGCAGCAACTATTATAAGATGCTCTGAAACTCTTCAGACACTGAGGGGGCACCAGAGGAGCAGACTACAAGA  639

ATG GCA CAC GCT ATG GAA AAC TCC TGG ACA ATC AGT AAA GAG TAC CAT ATT GAT GAA GAA GTG GGC TTT GCT CTG CCA AAT CCA CAG GAA  729
Met Ala His Ala Met Glu Asn Ser Trp Thr Ile Ser Lys Glu Tyr His Ile Asp Glu Glu Val Gly Phe Ala Leu Pro Asn Pro Gln Glu   30

AAT CTA CCT GAT TTT TAT AAT GAC TGG ATG TTC ATT GCT AAA CAT CTG CCT GAT CTC ATA GAG TCT GGC CAG CTT CGA GAA AGA GTT GAG  819
Asn Leu Pro Asp Phe Tyr Asn Asp Trp Met Phe Ile Ala Lys His Leu Pro Asp Leu Ile Glu Ser Gly Gln Leu Arg Glu Arg Val Glu   60

AAG TTA AAC ATG CTC AGC ATT GAT CAT CTC ACA GAC CAC AAG TCA CAG CGC CTT GCA CGT CTA GTT CTG GGA TGC ATC ACC ATG GCA TAT  909
Lys Leu Asn Met Leu Ser Ile Asp His Leu Thr Asp His Lys Ser Gln Arg Leu Ala Arg Leu Val Leu Gly Cys Ile Thr Met Ala Tyr   90

GTG TGG GGC AAA GGT CAT GGA GAT GTC CGT AAG GTC TTG CCA AGA AAT ATT GCT GTT CCT TAC TGC CAA CTC TCC AAG AAA CTG GAA CTG  999
Val Trp Gly Lys Gly His Gly Asp Val Arg Lys Val Leu Pro Arg Asn Ile Ala Val Pro Tyr Cys Gln Leu Ser Lys Lys Leu Glu Leu  120

CCT CCT ATT TTG GTT TAT GCA GAC TGT GTC TTG GCA AAC TGG AAG AAA AAG GAT CCT AAT AAG CCC CTG ACT TAT GAG AAC ATG GAC GTT 1089
Pro Pro Ile Leu Val Tyr Ala Asp Cys Val Leu Ala Asn Trp Lys Lys Lys Asp Pro Asn Lys Pro Leu Thr Tyr Glu Asn Met Asp Val  150

TTG TTC TCA TTT CGT GAT GGA GAC TGC AGT AAA GGA TTC TTC CTG GTC TCT CTA TTG GTG GAA ATA GCA GCT GCT TCT GCA ATC AAA GTA 1179
Leu Phe Ser Phe Arg Asp Gly Asp Cys Ser Lys Gly Phe Phe Leu Val Ser Leu Leu Val Glu Ile Ala Ala Ala Ser Ala Ile Lys Val  180

ATT CCT ACT GTA TTC AAG GCA ATG CAA ATG CAA GAA CGG GAC ACT TTG CTA AAG GCG CTG TTG GAA ATA GCT TCT TGC TTG GAG AAA GCC 1269
Ile Pro Thr Val Phe Lys Ala Met Gln Met Gln Glu Arg Asp Thr Leu Leu Lys Ala Leu Leu Glu Ile Ala Ser Cys Leu Glu Lys Ala  210

CTT CAA GTG TTT CAC CAA ATC CAC GAT CAT GTG AAC CCA AAA GCA TTT TTC AGT GTT CTT CGC ATA TAT TTG TCT GGC TGG AAA GGC AAC 1359
Leu Gln Val Phe His Gln Ile His Asp His Val Asn Pro Lys Ala Phe Phe Ser Val Leu Arg Ile Tyr Leu Ser Gly Trp Lys Gly Asn  240

CCC CAG CTA TCA GAC GGT CTG GTG TAT GAA GGG TTC TGG GAA GAC CCA AAG GAG TTT GCA GGG GGC AGT GCA GGC CAA AGC AGC GTC TTT 1449
Pro Gln Leu Ser Asp Gly Leu Val Tyr Glu Gly Phe Trp Glu Asp Pro Lys Glu Phe Ala Gly Gly Ser Ala Gly Gln Ser Ser Val Phe  270

CAG TGC TTT GAC GTC CTG CTG GGC ATC CAG CAG ACT GCT GGT GGA GGA CAT GCT GCT CAG TTC CTC CAG GAC ATG AGA AGA TAT ATG CCA 1539
Gln Cys Phe Asp Val Leu Leu Gly Ile Gln Gln Thr Ala Gly Gly Gly His Ala Ala Gln Phe Leu Gln Asp Met Arg Arg Tyr Met Pro  300

CCA GCT CAC AGG AAC TTC CTG TGC TCA TTA GAG TCA AAT CCC TCA GTC CGT GAG TTT GTC CTT TCA AAA GGT GAT GCT GGC CTG CGG GAA 1629
Pro Ala His Arg Asn Phe Leu Cys Ser Leu Glu Ser Asn Pro Ser Val Arg Glu Phe Val Leu Ser Lys Gly Asp Ala Gly Leu Arg Glu  330

GCT TAT GAC GCC TGT GTG AAA GCT CTG GTC TCC CTG AGG AGC TAC CAT CTG CAA ATC GTG ACT AAG TAC ATC CTG ATT CCT GCA AGC CAG 1719
Ala Tyr Asp Ala Cys Val Lys Ala Leu Val Ser Leu Arg Ser Tyr His Leu Gln Ile Val Thr Lys Tyr Ile Leu Ile Pro Ala Ser Gln  360

CAG CCA AAG GAG AAT AAG ACC TCT GAA GAC CCT TCA AAA CTG GAA GCC AAA GGA ACT GGA GGC ACT GAT TTA ATG AAT TTC CTG AAG ACT 1809
Gln Pro Lys Glu Asn Lys Thr Ser Glu Asp Pro Ser Lys Leu Glu Ala Lys Gly Thr Gly Gly Thr Asp Leu Met Asn Phe Leu Lys Thr  390

GTG AGA AGT ACA ACT GAG AAA TCC CTT TTG AAG GAA GGT TAA TGTAACCCAACAAGAGCACATTTTATCATAGCAGAGACATCTGTATGCATTCCTGTCATTACC 1914
Val Arg Ser Thr Thr Glu Lys Ser Leu Leu Lys Glu Gly xxx                                                                  403
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Fig. 2 Nucleotide sequence of C42 cDNA and deduced amino acid sequence of its protein product. A translation initiation consensus sequence (CCA/GCC, Ref. 25) was not observed.

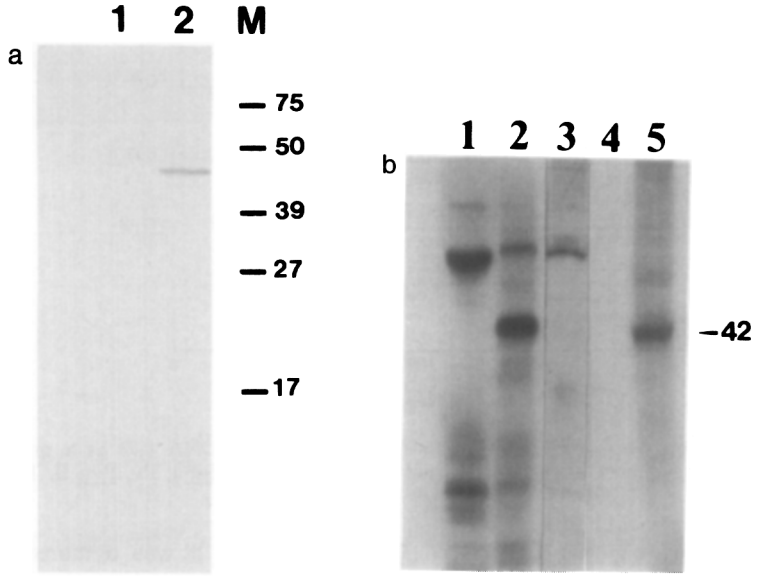


Fig. 3 (a) Immunoblotting of cell extracts from untreated (lane 1) and IFN-γ-treated (lane 2) FS-4 cells with a monoclonal anti-IDO antibody. (b) In vitro translation of C42 hybrid selected mRNA and immunoprecipitation with anti-IDO antibody. Poly(A)$^+$ RNA from HuIFN-γ-treated (300 units/ml, 24 hr) FS-4 cells was hybrid selected with C5-4 (or C42) cDNA plasmid or with pGEM-2 plasmid as a control essentially as described (19). The hybrid-selected mRNA was translated in vitro in a rabbit reticulocyte translation system (Promega) containing [^{35}S]methionine (50 μCi). The incubation mixtures were fractionated on a 10% SDS-polyacrylamide gel (23) either as such (lanes 1-3) or after immunoprecipitation with either anti-IDO antibody (lane 5) or anti-HLA-DRα antibody (as control, lane 4). Lanes 1 -3 show in vitro translation product with no added mRNA and mRNA hybrid selected with either C5-4 (C42) plasmid or pGEM-2 plasmid DNA, respectively. The gel was dried, soaked in Entensify (New England Nuclear) and exposed to Kodak XAR-5 film with DuPont Cronex intensifying screen. For immunoprecipition (lanes 4 & 5), aliquots of translation products (6 x 10^4 counts/min) were first denatured with 8 M urea at room temperature and precipitated with 5 volumes of acetone (-20°, 4 h). The protein precipitates were pelleted (12,000 g, 5 min.), air dried, redissolved in 30 μl of 0.1 N NaOH and then neutralized with 1 N HCl. To each sample, 8 μg of monoclonal antibody (either anti-IDO or anti-HLA-DRα antibody) in TNE buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mg/ml ovalbumin and 0.02% NaN_3) was added, and incubated for 1 hr at room temperature and then at 4°C overnight. Pansorbin slurry (30 μl, Calbiochem) was added and after a further incubation for 30 min. at room temperature, the samples were layered over 1 ml of washing buffer (100 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.3% bovine serum albumin) containing 10% sucrose in Eppendorf tubes. Pansorbin pellets were collected by centrifugation (12,000 g, 2 min.), washed once with 1 ml of washing buffer without sucrose, and eluted with 30 μl of SDS sample buffer (23) by boiling for 5 min.

presumably played an essential role in this transcriptional activation (17). It was subsequently reported that IDO purified from human placenta had an Mr ~40,000 (15), and that the induction of IDO by IFN-γ also required de novo synthesis of an intermediary protein (26; Beattie, R. and Gupta S. L., unpublished results). We therefore tested whether C42 cDNA may code for IDO.

In one set of experiments, we determined whether a monoclonal anti-IDO antibody (15) would immunoprecipitate the in vitro translation product of C5-4/C42-specific mRNA. The anti-IDO antibody specifically recognized, on Western blots, a polypeptide of Mr ~42,000 which was present in extracts of HuIFN-γ treated (Fig. 3a, lane 2) but not in extracts of untreated cells (lane 1). However, we found that this antibody was ineffective in

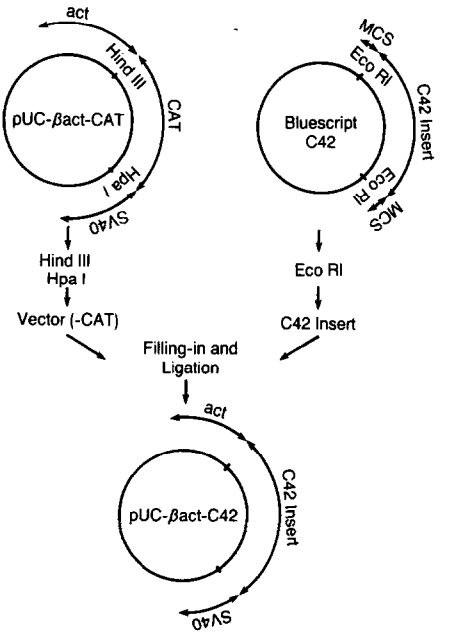
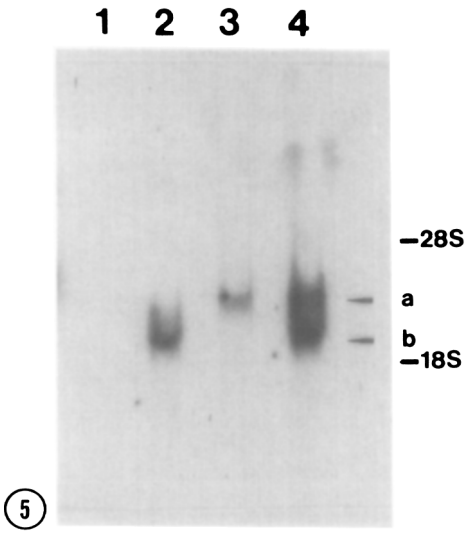


Fig. 4 Construction of β-actin-C42 expression plasmid. The C42 cDNA insert was isolated from Bluescript-C42 plasmid by digestion with EcoRI and ligated into pUC-βactin vector obtained form pUC-βactin-CAT plasmid by deletion of CAT structural sequence (by digestion with Hind III and Hpa I and fractionation by gel electrophoresis). The resulting plasmid (designated pUC-βactin-C42) was introduced into E. coli TB1 competent cells. Transformants harboring the C42 cDNA in right orientation with respect to the β-actin promotor were identified by restriction analysis of plasmid minipreps from several colonies. The plasmids used contained ampicillin resistance gene (not shown) which was used for selection of transformants.

immunoprecipitating the 42 kD polypeptide from HuIFN-γ-treated FS-4 cell extracts, which suggested to us that it may be directed against an internal epitope. If such extracts were denatured with 8 M urea before immunoprecipitation, the 42 kD polypeptide could be immunoprecipitated by the antibody (data not shown), thus supporting the above possibility. When C5-4/C42 hybrid selected poly(A)$^+$ RNA was translated in vitro, it directed the synthesis of a 42 kD polypeptide (Fig. 3b, lane 2), which was immunoprecipitated by anti-IDO antibody (lane 5) but not by anti-HLA-DRα antibody used as a control (lane 4). The 42 kD polypeptide product was not obtained when a control plasmid (pGEM-2) without a cDNA insert was used for hybrid selection (lane 3) or if no mRNA was added (lane 1).

As a second approach, we tested whether C42 cDNA could code for IDO activity. The C42 cDNA was cloned in an expression plasmid (pβact-CAT, Ref. 27) next to a chicken β-actin promotor (Fig. 4), and the plasmid construct was used together with pKOneo plasmid (28) containing neomycin resistance gene for co-transfection of GM00637D human fibroblasts. Stably transfected cells were selected for G418 resistance, propagated and tested for constitutive expression of (a) C42 specific RNA transcripts and (b) IDO activity. Fig. 5 shows that transfected cells constitutively expressed C42 specific RNA (lane 3) that was not observed in the parent cells (lane 1). This RNA transcript was significantly larger (~3 kb) than the IFN-γ-inducible C42 specific RNA product from cellular gene (~2.2 kb). This was apparently due to the fact that the vector contains a part of the actin transcribed sequence in addition



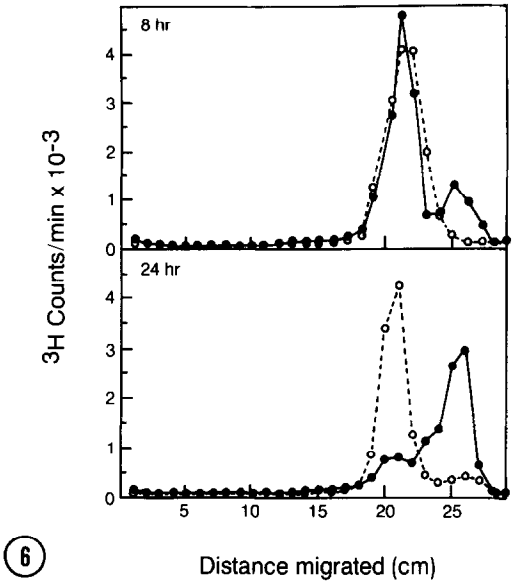


Fig. 5 Constitutive expressions of C42 specific RNA in GM00637D cells transfected with pUC-βact-C42 construct. GM00637D cells (obtained from Coriell Institute for Medical Research, Camden, NJ) were transfected with pUC-βact-C42 construct (see Fig. 4) together with pKOneo plasmid by calcium phosphate coprecipitation procedure essentially as described (29), and selected for resistance to G418 (500 μg/ml). Transfected cells (lanes 3 & 4) and untransfected parent cells (lanes 1 & 2) were incubated either without (lane 1 & 3) or with HuIFN-γ (lanes 2 & 4; 300 units/ml, 24 hr). Total cellular RNA was isolated and 15 μg of each was fractionated by formaldehyde-agarose gel electrophoresis as described (17), except that 10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA was used as the buffer instead. The gel was blotted onto nitrocellulose membrane, and the blot was probed with ^{32}P-labeled nick-translated C42 cDNA and autoradiographed. The constitutively expressed C42 specific RNA in transfected cells (lane 3) migrated slightly slower (indicated by a) than the IFN-γ-induced (2.2 kb) RNA (lanes 2 & 4, indicated by b).

Fig. 6 Constitutive expression of IDO activity in GM00637D cells transfected with pUC-βact-C42 plasmid. Confluent cultures of transfected (●) and untransfected (o) GM00637D cells (in 24 well tissue culture plates) were incubated in 250 μl of medium (1 part MEM: 3 parts Eagle's balanced salt solution, and 2.5% serum) containing ^{3}H-L-tryptophan (10 μCi) for either 8 hr or 24 hr. The culture media were collected, and incubated with trichloroacetic acid (5% final concentration) at 50°C for 30 min. The samples were centrifuged (12,000 g, 10 min), and the supernatants were analyzed by ascending paper chromatography in 0.1 M HCl as described (9). One cm. strips of the chromatograms were counted in a liquid scintillation counter for ^{3}H-radioactivity. The faster migrating material comigrated with L-kynurenine (data not shown).

to the actin promotor, and the SV40 splicing and polyadenylation sequences (27). Following HuIFN-γ treatment, both parent and transfected cells expressed C42 specific 2.2 kb RNA transcribed from the cellular gene (lanes 2 & 4, indicated by b), but as expected the 3 kb constitutively expressed C42 transcript in transfected cells was not stimulated (lane 4). Fig. 6 shows that cells transfected with C42 expression plasmid also constitutively expressed IDO activity as determined by degradation of ^{3}H-L-tryptophan. The parent cells showed little constitutive IDO activity (Fig. 6), but it could be induced by treatment with IFN-γ (not shown). These results showed that the cells transfected with C42 cDNA expression plasmid constitutively expressed C42 specific RNA and IDO activity, indicating that C42 cDNA represents a clone for IDO mRNA.

The induction of IDO by IFN-γ is of particular interest since in several cell types, IDO is induced strongly by IFN-γ but rather poorly by IFN-α or -β. This may be explained by the observation made earlier with the use of C5-4 cDNA clone as a probe (17) that the C5-4 (now = IDO) mRNA level was induced at least 10-fold higher by IFN-γ than by IFN-α, and whereas the induction by IFN-γ was sustained, the induction by IFN-α was only transient. Furthermore, it was observed that IFN-γ induced the transcription of the C5-4 (now = IDO) gene but de novo synthesis of some new protein was required (17), indicating that it played a vital role in this transcriptional activation and suggested that the putative required protein was induced by IFN-γ. Therefore, it appears that the expression of the C42 (IDO) gene by IFN-γ is brought about quite differently from the manner in which IFN-α and -β seem to activate cellular genes (30-36). The C42 cDNA will provide an important tool for studies on the regulation of IDO gene by IFN-γ, and the role of IDO in the biological activities of IFN-γ.

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