5. Peptides were synthesized as the carboxyl-terminal amide form by the solid-phase synthesis procedure [R. S. Hodges and R. B. Merrifield, *Anal. Biochem.* **65**, 241 (1975)]. Amino acid derivatives protected with the *t*-butyloxycarbonyl group in the α-amino position were obtained from the Protein Research Foundation (Osaka, Japan). The peptides were assembled by means of an Applied Biosystems Peptide Synthesizer Model 430, with ninhydrin testing at each step [V. Sarin *et al.*, *Anal. Biochem.* **117**, 147 (1981)]. The completed peptides were simultaneously deprotected and cleaved from the resin in anhydrous HF containing 10% (v/v) anisol [J. M. Stewart and J. D. Young, in *Solid Phase Peptide Synthesis* (Freeman, San Francisco, 1966), pp. 44 and 66]. Poor yields (<2%), of synthetic product were obtained when *t*Boc-*N*-tosyl-im-histidine was used, whereas yields of approximately 17% were obtained with the use of *t*-butyloxycarbonyl-*N*-dinitrophenol-im-histidine. Dinitrophenol deprotection was done prior to HF cleavage by treating the resin with 2-mercaptoethanol 20% (v/v) and diisopropyl ethylamine 2% (v/v) in dimethylformamide [S. Kent and I. Clark-Lewis, in *Synthetic Peptides in Biology and Medicine*, K. Alitalo, P. Partarmen, A. Vaheri, Eds. (Elsevier, Amsterdam, 1985), pp. 29–57]. Peptides were extracted from the resin with 60% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid solvent, subjected to rotary evaporation, and lyophylized from water. The peptides were purified by sequential ion-exchange chromatography (either SP-Sephadex or CM-Sephadex) and preparative low-pressure reversed-phase chromatography (25 by 400 mm column, C18, 250 Å, 35 to 70 μm Amicon resin) with an acetonitrile gradient in the presence of 0.1% (v/v) trifluoroacetic acid. The synthetic PTHrP(1–34) migrated as a major peak coincident with PTH-like biological activity. Biologically active synthetic PTHrP(1–34) was obtained from three independent syntheses. Amino acid analyses of purified peptides were in good agreement with theoretical values. For example, the average of two analyses of 24-hour hydrolysates of PTHrP(1–34) yielded the following number of residues: Asx (2.03), Thr (0.98), Ser (2.15), Glu (3.46), Gly (1.52), Ala (3.29), Val (1.10), Ile (2.86), Leu (4.96), Phe (2.06), His (4.63), Lys (2.14), and Arg (2.64).
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18 September 1987; accepted 3 November 1987

Identification of the Iron-Responsive Element for the Translational Regulation of Human Ferritin mRNA

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Regulated translation of messenger RNA offers an important mechanism for the control of gene expression. The biosynthesis of the intracellular iron storage protein ferritin is translationally regulated by iron. A *cis*-acting element that is both necessary and sufficient for this translational regulation is present within the 5′ nontranslated leader region of the human ferritin H-chain messenger RNA. In this report the iron-responsive element (IRE) was identified by deletional analysis. Moreover, a synthetic oligodeoxynucleotide was shown to be able to transfer iron regulation to a construct that would otherwise not be able to respond to iron. The IRE has been highly conserved and predates the evolutionary segregation between amphibians, birds, and man. The IRE may prove to be useful for the design of translationally regulated expression systems.

IN THE PAST FEW YEARS, INTENSIVE scientific efforts have led to fascinating insights into transcriptional regulatory mechanisms (*1*). In contrast, there is only a limited list of eukaryotic genes for which translational regulation of messenger RNA (mRNA) utilization has been reported (*2*). As one of the earliest examples, Munro and his colleagues demonstrated that the biosynthesis of the intracellular iron storage protein ferritin is regulated by iron in the absence of alterations in mRNA levels and proposed that this regulation took place at the translational level (*3*). It was shown that cytoplasmic ferritin mRNA underwent a redistribution from an inactive ribonucleoprotein pool to translationally active polyribosomes after iron induction (*4*). Ferritin is a particularly useful model to study the translational regulation of a eukaryotic gene. The protein is highly conserved in all eukaryotic cells (*5*), and the regulation of its biosynthesis by iron is rapid, reversible, and covers more than a 100-fold range (*6, 7*). We have recently shown that the expression of the human ferritin H chain is translationally regulated by iron in transiently transfected and stably transformed murine fibroblasts (*7, 8*). The presence of an iron-responsive cis-acting element in the translated 5′ leader region of the ferritin mRNA was necessary to achieve regulation. Furthermore, the presence of the complete 5′ leader sequence proved to be sufficient to regulate the expression of a chloramphenicol acetyl transferase (CAT) construct that was expressed from the ferritin promoter and encoded a ferritin-CAT fusion protein. Quantitative S1 nuclease protection assays demonstrated that the resulting iron regulation of CAT occurred in the absence of any changes in the level of specific mRNA (*8*). The purpose of the present study was to identify the iron-responsive element (IRE) and to begin to characterize its structure and function.

Our initial strategy was to localize the IRE by progressive deletional analysis. Figure 1A shows the nucleotide sequence of the 5′ leader region of the human ferritin H-chain complementary DNA (cDNA). Fragments containing the ferritin promoter and portions of this 5′ leader region were generated by Bal 31 digestion from a parent construct, pFPL CAT (*8*). The 3′ end points of these fragments as well as the nature of an internal deletion were determined by DNA sequencing (Fig. 1A) (*9*). Expression constructs containing the minimally deleted L1 (Δ−1/−5) and maximally deleted L5 (Δ−1/−206) ferritin promoter plus 5′ leader fragments were generated with either CAT (*10*) or human growth hormone (hGH) (*11*) protein coding regions. Iron responsiveness of these constructs was determined by treating transient transfectants with hemin (H) as an iron source or desferrioxamine (D) as an iron chelator and determining the level of CAT activity or the hGH level relative to an identical untreated control (C) transfectant. The H/D ratio for each deletion construct indicates the range of iron regulation that was observed for that construct under the experimental conditions. This ratio and thus the iron responsiveness observed with construct L1-CAT (Fig. 1B, inset) was identical to that seen with the parent construct, pFPL CAT (*8*). In the construction of L1-CAT, the ferritin ATG present in pFPL CAT was removed, leaving the CAT ATG as the translation start codon. The observation of identical iron

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responsiveness of L1-CAT and pFPL CAT indicates that the exact nucleotide context surrounding the translation start site of the ferritin mRNA is not critical for the function of the IRE. When almost all of the ferritin leader sequence was deleted, the resulting construct (L5-CAT) was unresponsive to iron perturbations (Fig. 1C, inset).

Fragments L1 and L5 were also utilized to direct the expression of the secretory protein hGH (Fig. 1, B and C). The iron-dependent range of biosynthetic regulation by the IRE in L1-GH appears greater than that suggested by the L1-CAT assay results (Fig. 1B), even though the identical ferritin promoter plus 5′ leader fragments were utilized. Indeed, the calculated H/D ratio for L1-CAT is 6.4, while that for L1-GH over the last 16 hours of sampling is 11.5. This difference in ratio is most likely a consequence of the fact that the secretory rate of hGH more closely reflects the biosynthetic rate of the indicator protein than does the accumulation of cytoplasmic CAT enzymatic activity. The ability to regulate the expression of both a cytoplasmic and a secreted protein implies that the IRE can function in mRNAs translated on both free and endoplasmic reticulum–associated ribosomes. These results with constructs in which fragments L1 and L5 direct heterologous gene product expression indicate that an IRE is localized between −206 and −5 of the 5′ leader sequence of the ferritin H-chain mRNA.

The deletion constructs utilizing the leader fragments shown in Fig. 1A were transiently expressed and assayed for CAT activity (Table 1). Additional removal of nucleotides −6 to −142 (constructs L2-CAT and L3-CAT) of the ferritin leader sequence had little appreciable effect on the degree of iron regulation when compared to the range displayed by L1-CAT. Thus, the absolute distance between the IRE and the translation initiation codon need not be fixed in order to observe the effect of the IRE. In contrast, when the deletion extended from −1 to −186 or −206 (constructs L4-CAT and L5-CAT, respectively), iron regulation was completely abolished. To investigate whether the critical region for iron regulation is localized between −142 and −186, a construct was generated that contained an internal deletion of bases −151 to −186 from L1-CAT [see Fig. 1A and (*9*)]. This construct, L1(Δ−151/−186)-CAT, did not respond to iron perturbations (Table 1). These results imply that the region −186 to −151 contains an IRE for the translational regulation of ferritin biosynthesis. The absolute amount of CAT activity per milligram of cellular protein varied for the same construct from precipitate to precipitate. All comparisons of the effect of iron on CAT activity were performed by aliquoting the DNA from single precipitates. Despite changes in the absolute CAT activity with different precipitates, the H/D ratio for any given construct was independent of the total level of CAT expression and was highly reproducible from experiment to experiment as shown in Table 1.

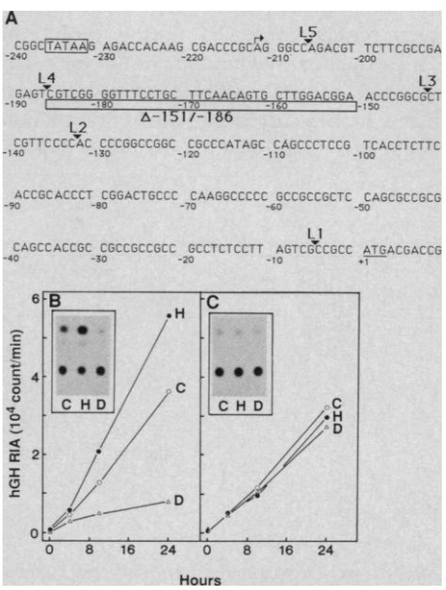

Fig. 1. Partial nucleotide sequence of the deletion fragments of the 5′ noncoding leader sequence from human ferritin H chain (A), and analysis of iron-dependent regulation of indicator gene product expression by means of minimally and maximally deleted leader fragments (B and C). (**A**) The translation start site is numbered +1; the nucleotide sequences of the 5′ noncoding region are counted 3′ to 5′ from the ATG, starting at −1; the transcription start site (*8, 19*) is marked with an arrow (−212); and the TATAA box is outlined. The 3′ boundaries of deletion fragments (L1 to L5) are marked by arrowheads, and the region deleted in the single internal deletion fragment is indicated by the open bar (Δ−151/−186) (*9*). (**B** and **C**) Enzymatic activity of CAT expression (insets) and production of hGH are depicted for constructs directed by the minimally deleted L1 fragment (B) or maximally deleted L5 fragment (C) as a function of iron level modulation. In the measurements of CAT activity, the upper and lower spots represent acetylated and unacetylated chloramphenicol, respectively. C, assay of control transfectants; H, assay of transfectants treated with hemin as an iron source; D, assay of transfectants treated with desferrioxamine as an iron chelator. CAT assays were performed with equivalent fractions of a cell lysate of transiently transfected murine fibroblasts. Lysates from untransfected cells assayed similarly displayed no detectable CAT activity. The hGH assays were performed on culture supernatant of transiently transfected cells with a commercially available hGH radioimmunoassay kit (*20*).

Table 1. Relative iron regulation of CAT activity imparted by deletion fragments of the ferritin 5′ leader. Deletion leader fragments as indicated in Fig. 1A were used to construct CAT plasmids as described (*9*). Transient transfections and CAT assays (a) were performed with triplicate transfections that were either untreated or treated with 100*M* hemin (H) or 100 μ*M* desferrioxamine for 20 hours prior to preparation of cell lysates for determination of CAT activity (*10*). Iron responsiveness of L1-CAT activity was determined as a positive control in all experiments. Relative iron regulation was determined by comparing the range of observed iron regulation as expressed by the H/D ratio (see text) for each construct to the H/D ratio for L1-CAT, with the relative iron regulation level of L1-CAT defined as 100. Mean levels of iron regulation for each construct ± SEM are shown and the number (*n*) of independent triplicate transfections for each construct are indicated in parentheses.

Plasmid construct	Deletion	Relative iron regulation (*n*)
L1-CAT	−1/ −5	100
L2-CAT	−1/−132	87.6 ± 10.8 (3)
L3-CAT	−1/−142	85.2 ± 4.5 (3)
L4-CAT	−1/−186	0.2 ± 1.8 (2)
L5-CAT	−1/−206	3.8 ± 3.5 (7)
L1(Δ−151/−186)-CAT	−151/−186 and −1/−5	2.9 ± 3.3 (3)

Computer modeling of the mRNA secondary structure (*12*) of the core region suggested by our deletional analysis predicts a stem-and-loop structure (Fig. 2). This feature of the IRE may be of functional significance. The presence of stable stem-and-loop structures in other mRNAs can drastically reduce the rate of translation, possibly by interfering with "ribosome scanning" (*13*). In the case of the ferritin IRE, trans-acting factors could conceivably alter the stability of the stem-and-loop structure through their interaction and thereby regulate translation (*7*).

To prove the identity of the IRE suggested by the deletion studies we designed and synthesized two complementary synthetic oligodeoxynucleotides (26mers). They encompassed the core region of the sequences that were implicated by the Bal 31 deletion analysis and provided bilateral Bam HI linker sites (Fig. 2). These two oligonucleotides were annealed and cloned into L5-GH, a construct unresponsive to iron, at the Bam HI site located between the promoter plus remaining 5′ leader and the indicator gene (Fig. 1C). Figure 3 shows the hGH assay results obtained from murine fibroblasts that were stably transformed with the parent plasmid control (L5-GH) and the

newly constructed plasmid L5(+26mer)-GH, which contains a single insert in the physiological 5′ to 3′ orientation with the predicted nucleotide sequence. The insertion of the oligonucleotide reconstituted iron responsiveness, demonstrating that the oligonucleotide can function as an IRE. Northern blot analysis confirmed that iron regulation occurred in the absence of iron-induced changes in the level or size of the hGH mRNA (*14*). Analysis of different stable cell lines expressing L5(+26mer)-GH showed that the quantitative responsiveness to iron is independent of the level of gene expression over a more than 10-fold range in basal hGH expression (*14*).

The identification of an IRE and the successful reconstitution of iron regulation by insertion of a synthetic oligonucleotide offers the chance to investigate the molecular mechanisms underlying the translational regulation of ferritin biosynthesis by mutagenesis. Two deletion mutants shown in Fig. 2 (Δ−165 and Δ−168) identify a functionally critical region of the IRE. In both cases, cytidine residues have been omitted from defined positions of two synthetic oligonucleotides. The Δ−165 represents a deletion of the most 5′ nucleotide of the loop of the proposed stem-and-loop structure. In contrast, Δ−168 lacks the cytidine residue in the middle of the stem and therefore may interfere with the ability of the mRNA to form such a base-paired structure. Both deletions completely abolish the iron response (*14*). We cannot distinguish whether the functional loss results from the change in the primary nucleotide sequence or an alteration of the secondary structure, or both. We conclude from this experiment that both deletions affect a functionally critical region of the IRE. Comparison of the core region of the human ferritin H-chain cDNA leader sequence (which contains the IRE) with the leader sequences of the human ferritin L-chain cDNA (*15*) and the cDNA sequences of ferritins from other species (*16, 17*), reveals that the core region of the predicted stem-loop structure has been highly conserved during evolution. This sequence predates the evolutionary segregation between amphibians, birds, and man which occurred more than 300 million years ago (*17*). Shaded bases in Fig. 2 indicate a region that is 100% identical to the IREs found in all complete ferritin cDNA sequences published thus far.

We have previously shown that deletion of nucleotides −30 to −186 of the 5′ leader region of the ferritin mRNA results in a complete loss of iron regulation of the ferritin gene product (*8*). Although we have transferred iron regulation to heterologous gene products, the range of iron regulation appears to be less (by about fivefold) than we observe with the endogenous gene. In particular, the effect of added iron (in the form of hemin) is less in the hybrid constructs than in the native gene. The basis for this apparent difference is unknown.

In addition to providing insights into the molecular mechanisms underlying the regulation of translation in eukaryotic cells, we believe that the IRE can potentially serve to develop a new class of regulatable expression vectors. We have shown that the IRE can regulate the expression of both cytosolic and secretory proteins in transfected murine fibroblasts. Insertion of the IRE into the 5′ leader sequence of a cDNA to be expressed should allow accumulation of mRNA without substantial translation under conditions of iron deprivation. Subsequently, the addition of iron donors to the culture medium should result in a dose-dependent increase in translation of the specific protein.

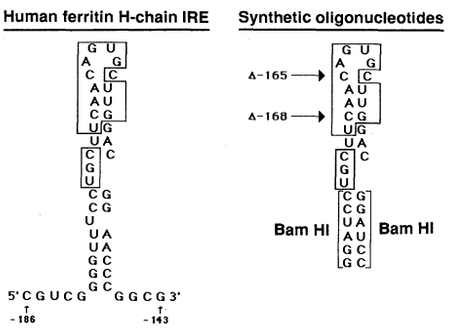

Fig. 2. Sequence and computer-modeled secondary structure of the human ferritin H-chain IRE encoded mRNA and synthetic oligonucleotide encoded mRNA. Computer-modeled (*12*) secondary structure of the mRNA encoded by bases −186 to −143 of the ferritin H-chain 5′ leader was generated as a preferred possible secondary structure for this region when analyzing the mRNA sequence encoded by nucleotides −212 to −92. Two complementary oligonucleotides were synthesized (Midland Certified Reagent Company, Midland, Texas) that were provided with bilateral Bam HI linker sites, annealed, phosphorylated, and cloned into the Bam HI sites of the pUC 18 polylinker situated between known 5′ leader fragments and the CAT Genblock of previously constructed plasmids (see Fig. 1). Recombinants were selected by colony hybridization; copy number, orientation, and nucleotide sequence of the inserts were determined directly from the double-stranded plasmids by a modification of the dideoxy chain termination method (*18*). The coding synthetic oligonucleotide contributed 26 additional bases to the plasmid-generated mRNA of the parent constructs, and 24 of 26 bases corresponded to and were 100% identical to bases −176 to −153 of the native IRE mRNA, as shown on the left. The one 5′-most base and five 3′-most bases shown are generated by the Bam HI polylinker site of the parent plasmids and provide possible additional stability to the secondary structure of the mRNA. Single base deletion mutations in the sequence of the synthetic oligonucleotide were isolated and are indicated (Δ−165 and Δ−168) using numbering which corresponds to the native IRE sequence. Shaded bases represent those showing 100% identity with cDNA sequences of human ferritin L chain, rat ferritin H and L chains, and bullfrog and chicken ferritin H chains (*14–16*).

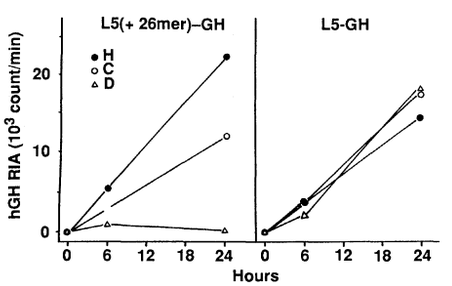

Fig. 3. A synthetic nucleotide homologous to the ferritin 5′ leader IRE can reconstitute iron responsiveness. The hGH assays were done with culture supernatant of stably transformed cells left untreated (C) or treated with hemin (H) or desferrioxamine (D) as described above (see Fig. 1 and Table 1). Assays are shown for the non-iron-responsive parent plasmid L5-GH and for the plasmid containing a single copy, correctly oriented synthetic oligonucleotide of the predicted sequence −L5(+26mer)-GH. Stable cell lines were established as described (*18*).

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9. The parent construct, pFPL CAT (*8*), was cut 12 bp 3′ of the ATG translation start codon with the restriction endonuclease Bam HI and was subsequently subjected to exonuclease Bal 31 digestion for varying lengths of time. The reaction was stopped and recessed termini were filled in with the Klenow fragment of DNA polymerase I [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: a Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. Subsequently, fragments containing the ferritin promoter and the remainder of the 5′ leader sequence were released with the restriction endonuclease Sac I and isolated after electrophoresis in a 2.0% agarose gel. These fragments were subcloned into the Sac I–Sma I sites of the replicative form of the M13 mp18 vector and sequenced by dideoxy chain termination (*18*) to determine the 3′ end point resulting from the Bal 31 deletion. Suitable deletion fragments were recovered from the replicative form by first

opening the M13 construct at the 5′ end of the fragment with an Eco RI digest, filling in recessed termini with the Klenow fragment of *Escherichia coli* DNA polymerase, then releasing the fragment with a Bam HI digest. These fragments were then subcloned directionally into vectors just 5′ of a previously inserted indicator gene, either into the Sma I–Bam HI polylinker sites of a pUC18 vector containing the CAT GenBlock (Pharmacia) in the Hinc II site of the polylinker, or into the Hinc II–Bam HI polylinker sites of a pUC12 vector containing a promoterless hGH gene at the Bam HI–Eco RI sites of the polylinker (*11*). An internal deletion leader fragment which has bases −186 to −151 removed from fragment L1 was generated by performing Bal 31 digestion of L1-CAT from the 5′-most end of the ferritin promoter and extending the digestion into the region corresponding to the 5′ noncoding region of the ferritin mRNA. Release, M13 cloning and sequencing, and subsequent fragment isolation were performed as above. A fragment corresponding to base pairs −150 to −6 was identified, isolated, and subcloned in the proper orientation into L4-CAT at polylinker sites between the L4 fragment and the CAT GenBlock. The net effect was the construction of L1-CAT with an internal deletion of base pairs −186 to −151.

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20. CAT assays on transient transfectants was performed as described (*8*). Assays for hGH production were performed as follows. Murine B6 fibroblasts were transfected in triplicate with 30 μg of plasmid DNA by calcium phosphate precipitation (*8*) and washed twice with media after 16 hours. Transfectants were then cultured at 37°C for 24 hours in Dulbecco's modified essential media before changing to fresh media and adding hemin (100 μ*M*) or desferrioxamine (100 μ*M*) or leaving as untreated control. The time of addition of agents was designated time 0, and aliquots of media were taken and stored at −20°C. Additional aliquots were taken at time points 4, 10, and 24 hours. Levels of hGH as a function of time and treatment were then determined by radioimmunoassay (Nichols Institute, San Juan Capistrano, CA), and the absolute ^{125}I counts as determined by radioimmunoassay plotted as a function of time.
21. We thank H. Masukata, NIH, for performing the structural RNA analysis; J. Casey and D. Koeller, NIH, for valuable discussions and assistance with sequence homology analysis; and E. Perry for her excellent assistance in manuscript preparation. M.W.H. had a fellowship from the Deutsche Forschungsgemeinschaft and S.W.C. was supported by the Dermatology Branch, National Cancer Institute, NIH.

17 August 1987; accepted 20 October 1987

Phagocytosis of *Candida albicans* Enhances Malignant Behavior of Murine Tumor Cells

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Murine tumor cells were induced to phagocytize either *Candida albicans* or group A streptococcal cells. The presence of microbial particles within the tumor cell cytoplasm had no effect on in vitro tumor cell growth. However, when *Candida albicans*–infected tumor cells were injected into syngeneic mice, they formed tumors that grew faster, invaded the surrounding normal tissue more rapidly and metastasized more rapidly than control tumor cells. Tumor cells infected with group A streptococcal particles did not grow faster or show increased malignant behavior. These data indicate that the in vivo behavior of malignant tumor cells can be modulated by microbial particles, which are often present in the microenvironment of the growing tumor.

PHAGOCYTOSIS OF PARTICLES THE size of red blood cells, mammalian cell nuclei, and microorganisms can be experimentally induced in nonleukocytic cells by a variety of manipulations (*1–4*). Routine histological and cytological examination of tissues and cells taken from human tumors also frequently shows the presence of intracytoplasmic particles (*5–11*), suggesting that this phenomenon is not just an in vitro artifact. The presence of intracytoplasmic bacteria in hyperplastic and dysplastic gut epithelium of several species has been observed and viable bacteria have been isolated from the epithelial cells (*12–20*). These findings suggest that some forms of adenocarcinoma of the ascending colon may have an infectious component to their etiologies (*12, 13*). How the presence of intracytoplasmic infectious agents might influence the behavior of the host cell is difficult to assess because there have been no experimental models with which to study this phenomenon. In the present study, we have induced the intracytoplasmic accumulation of microorganisms by murine tumor cells and have examined the effect on the in vivo biological properties of the cells.

A line of murine fibrosarcoma cells designated as 1.0/L1 was used in our studies. These cells were isolated in our laboratory from a 3-methylcholanthrene–induced tumor in a C57B1/6 mouse (*21*). They grow in monolayer culture on RPMI 1640 culture medium supplemented with 10% fetal bovine serum. When injected subcutaneously into syngeneic mice, they form progressively growing tumors that invade locally and metastasize to the lungs. Micrometastases first become visible at approximately 30 to 40 days after subcutaneous tumor injection, at which time the primary tumors have reached a mean diameter of 10 mm or more.

Phagocytosis of microorganisms [heat-killed group A streptococci (strain C203S, M protein–negative) or *Candida albicans*] by the murine tumor cells was induced as described (*2*). This procedure involved preparing suspensions of heat-killed cells at 1×10^7 cells per milliliter of phosphate-buffered saline (PBS) and opsonizing the microbial cells by the addition of 1 mg of histone per milliliter (type II-A, Sigma Chemical Co., St. Louis, Missouri). After 30 minutes of incubation, the microbial cells were washed repeatedly to separate them from the unbound histone. The opsonized microorganisms were then added to monolayers of tumor cells in serum-free RPMI 1640 medium at a ratio of 10:1 for the *C. albicans* and 50:1 for the streptococci. Although unopsonized microorganisms did not attach to the tumor cells, the histone-treated cells rapidly attached. More than 95% of the added cells were firmly attached to the tumor cell monolayer within 15 minutes. It could be seen microscopically that the opsonized cells did not attach to the plastic dish itself. Morphometric techniques were used to determine the kinetics of microbial cell uptake by the tumor cells and to quantitate the distribution of microbial cells within individual tumor cells. Uptake of the opsonized microbial particles began within 6 hours after being added to the tumor cell monolayer and was essentially complete by 36 hours. Approximately 70% of the tumor cells contained intracellular particles ranging in number from 1 to 10 yeast particles per cell and 1 to 25 bacterial particles per cell.

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