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DEVELOPMENT OF EXPRESSION VECTORS FOR TRANSGENIC FISH

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Genetic alteration of fish is important for aquatic biotechnology as well as for investigating molecular interactions that occur during vertebrate development. The numerous, large, transparent, and externally fertilized eggs of many fish species make them ideally suitable for genetic manipulation, especially for production of transgenic animals. Genetic engineering of fish requires suitable expression vectors. Accordingly, we developed two fish expression vectors, FV-1 and FV-2, which contain the proximal promoter and enhancer regulatory elements of the carp β-actin gene and the polyadenylation signal from the salmon growth hormone gene. The two fish expression vectors were tested in microinjected fish eggs and in tissue cultured fish and mammalian cells. These two "all-fish" expression vectors should be useful for genetic engineering of fish and have been used with growth-enhancing genes in transgenic fish.

Aquatic biotechnology, aquaculture, is a significant factor in worldwide fisheries production, which has grown more than 30% over the past seven years to about 21 billion pounds. In 1986 the total fish catch was about 91 million metric tons of which about one third accounted for $24 billion in international trade[1]. In addition to its obvious economic importance, aquaculture provides a critical portion of human nutrition due to the efficiency of food conversion in fish. Food conversion ratios (nutrition input relative to fish mass) of 1.5 to 1 can be achieved in fish compared to 12 to 1 for grass-fed cattle and 1.7 to 1 for highly improved strains of chicken[1]. The anticipated growth of aquaculture will require genetic improvement programs.

Besides their importance as a source of nutrition for humans and their economic value, fish are useful as model systems for biological research[2]. They represent the largest and most diverse group of vertebrates and can be easily raised in the laboratory, often less expensively than other vertebrates. On a periodic basis many fish species produce numerous eggs that are generally large and externally fertilized. Homozygous diploid fish have been produced and the phenotype can be controlled by hormones[2,3]. These features make them suitable for genetic manipulation. External fertilization and development of eggs in most species permit easy introduction of foreign genes into fish for the production of transgenic animals. Transparent fish eggs are ideal for the study of ongoing embryonic development. In addition, some fish such as zebrafish *(Brachydanio rerio)*, have short life cycles of several months and therefore can be used as models for genetic analysis[4].

Because fish are an important source of nutrition for humans, introduction of growth-enhancing genes into fish was initiated following the demonstration of growth augmentation in mice after transfer of rat growth hormone genes[5,6]. The introduction of foreign genes into fish requires suitable expression vectors with appropriate regulatory elements for control of transgene expression. The promoters used to date in transgenic fish studies have been from the mouse metallothionein gene[7–11], the Rous sarcoma virus (RSV)[12], and the simian virus 40 (SV40)[13]. These promoters show a wide range of tissue expression, but all have disadvantages. The metallothionein promoter shows its highest expression following induction by toxic, heavy metals or glucocorticoids. These promoters plus those from viral sources are tainted for use in aquaculture by their association with metabolic poisons or tumor-inducing sequences.

Accordingly, we began development of vectors containing appropriate regulatory sequences of piscine origin for expression of any gene or cDNA of interest for use in biological research and aquaculture. Such an "all-fish expression vector" should include a strong promoter, an intron and a polyadenylation signal. Here we report the construction of several expression vectors that express the bacterial chloramphenicol acetyl transferase reporter gene at high levels. The "all-fish" vectors developed here should be suitable for production of transgenic fish for scientific and aquacultural purposes.

RESULTS

Strategy. To develop "all-fish" expression vectors, we used the available carp β-actin regulatory elements[14,15] to drive expression because this promoter is strong and has a wide range of tissue expression. The complexity of this promoter enabled us to make vectors with variable levels of expression. Furthermore, the high evolutionary conservation of the identified regulatory sequences of β-actin genes strongly indicated that the expression vector employing these elements should be functional in most, if not all, species of fish. We employed the poly(A) signal from the chinook salmon growth hormone gene[16] to assure controlled termination of the mRNAs. The bacterial chloramphenicol acetyl transferase (CAT) gene was used as a marker gene because its expression is easily monitored and it is not endogeneous in fish. Fish cells have negligible background CAT activity. To evaluate the relative expression potential of the fish vectors, we compared their expression with other widely used vectors, including the pRSVcat and pSV2cat expression vectors[17,18], and the pHSPcat, inducible vector containing the heat shock promoter (Wu, personal communication). We

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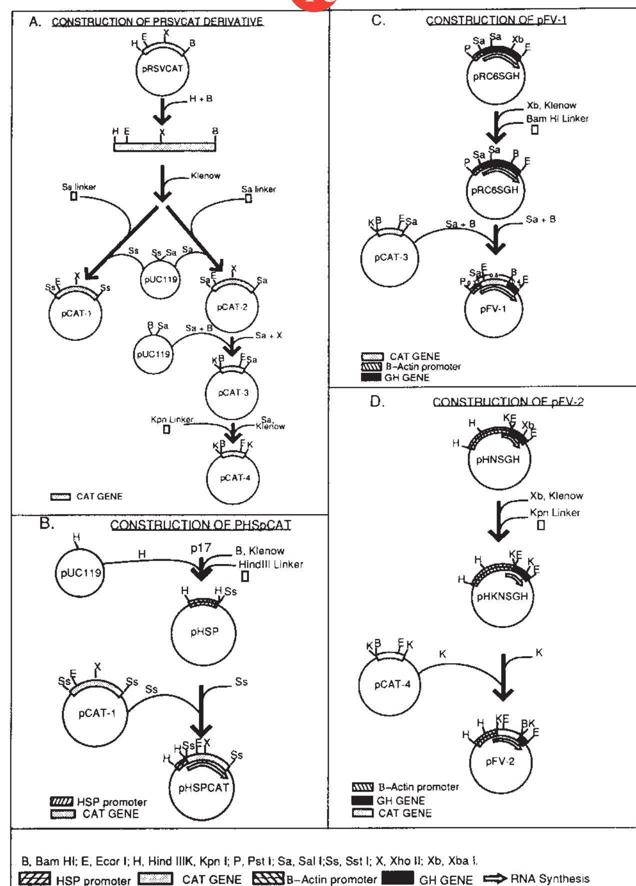

FIGURE 1 Construction of fish expression vectors. Detailed steps for making the constructs are described in the text. (A) Construction of CAT plasmids for promoter insertions. (B) construct of pHSPcat. (C) construct of pFV-1. (D) construct of pFV-2.

TABLE 1 Transgene expression in tissue-cultured cells. Constructs were transfected into mouse L cells and carp EPC cells as described. Cells were harvested and CAT analysis was performed 60 hours after transfection. Four plates were used for each construct and the levels of CAT expression were averaged. The numbers were normalized to that of pFV-2. In mouse L cells and carp EPC cells, 100% corresponds to 0.1 unit and 0.02 unit of CAT activity, respectively, as compared with commercial (Sigma) preparations of CAT analyzed under identical conditions[15].

Constructs	Mouse L cells	Carp EPC cells
pCAT-1	0.1	0.1
pRSVcat	18	41
pHSPcat	4	6
pHSPcat (HS)	20	19
pSV2cat	16	6
pFV-1	14	6
pFV-2	100	100

transferred the experimental constructs, in the form of supercoils, into both transfected cells and microinjected zygotes. In this way we sought to avoid complications in the assays due to differential rates of integration, sites of integration, and other modifications of the transgenic DNA that might have occured with linearized constructs and thus interferred with our conclusions. The supercoiled constructs are transcriptionally active and more stable in cells and zygotes in the unintegrated state than their linearized homologues[19].

Construction of piscine expression vectors. To make the vectors with the CAT marker gene, we first made a series of derivatives of pRSVcat[17] suitable for promoter insertion (Fig. 1A). In pRSVcat, the CAT gene, intron of SV40 and the early gene poly(A) signal sequence are located in a 1.6 kb HindIII/BamHI fragment. The first fish expression vector, pFV-1 (Fig. 1C), was constructed by inserting the SalI/BamHI fragment of pCAT-3 into the SalI/BamHI sites of pRC6sGH[15]. Similarly, a second fish expression vector pFV-2 (Fig. 1D), was constructed by inserting the KpnI CAT gene fragment of pCAT-4 into pHNsGH[15] between the KpnI site and XbaI site in which the XbaI sites had been modified to a KpnI site by use of linkers. The difference between pFV-1 and pFV-2 is the inclusion in pFV-2 of a non-coding first exon, the first intron, and the 5′ end of the second exon from the carp β-actin gene. pFV-1 would be suitable for expression of whole genes with introns whereas pFV-2 should be capable of expressing both cDNA sequences as well as intron-containing genes. Furthermore, pFV-2 has increased expression potential since a positive regulatory element is contained in the first intron of the carp β-actin gene[15]. We also constructed an expression vector with an inducible promoter from the human heat shock protein 70 (*hsp*) gene (Fig. 1B). The *hsp* promoter was derived from plasmid p17[20]. The pHSPcat was constructed by inserting the CAT gene, on an SstI fragment from pCAT-1, into the SstI site of pHSP.

Analysis of expression in transfected cells. CAT constructs with five different promoters were first tested for expression in transfected cell lines. pRSVcat, pSV2cat, and pHSPcat each contain a promoter, the bacterial CAT gene, and both the SV40 *t*-antigen intron and early region poly(A) signal. pFV-1 and pFV-2 both contain the CAT gene and the poly(A) signal sequence of the chinook salmon growth hormone gene. pFV-1 contains 286 bp of the carp β-actin gene, including the basic promoter and part of the non-translated first exon. pFV-2 contains 1.1 kb of upstream sequence, the entire first exon, the entire first intron, and the 5′ proximal portion of the second exon of the carp β-actin gene. These constructs were introduced into mouse L cells by DEAE-dextran mediated transfection[21], and into carp epithelial cells (EPC) by calcium phosphate-mediated transfection[22,23]. In mouse L cells, pFV-2 was expressed at the highest level, at least 5 times higher than pSV2cat, pRSVcat, pFV-1, or pHSPcat following heat shock induction (Fig. 2 and Table 1).

The expression of these constructs in the fish cell lines was qualitatively similar to that in mouse L cells, except that pRSVcat expressed CAT at about twice the rate as in L cells, whereas pSV2cat and pFV-1 expressed CAT at a lower level (Table 1). We have shown previously by S1 nuclease analysis that the transcriptional initiation site and RNA splicing sites are not affected in our expression constructs[15]. For ease of comparison, the values in Table 1 are normalized to pFV-2, the strongest expression vector. The assays were repeated four times; repeated trials with a given construct varied by no more than 10%. Based on CAT activity per microgram of protein cell extract, pFV-2 was approximately 5-fold less active in carp EPC cells than in L cells. The lower levels of CAT activity in fish cells was mainly due to a much lower rate of DNA transfer by transfection. Either a dimethyl sulfoxide (DMSO) or a glycerol shock, an important step for transfection of mammalian and avian cells, was lethal in fish cells and was therefore not included. Omission of the DMSO or glycerol shock lowers the transfection ratios (Liu, unpublished observations). Additionally, the lower temperature of incubation of the EPC cells (28°C) compared to that of L cells (37°C) may have had an effect on overall levels of CAT gene expression.

Analysis of expression in transgenic fish. The results from the tissue-cultured cell lines indicated that the two

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fish expression vectors were responsive to piscine transcriptional factors. To test the usefulness of the fish expression vectors in transgenic animals, the DNA constructs were microinjected into fertilized embryos of zebrafish and samples were randomly taken from each microinjected batch each day after microinjection for ten days. CAT assays were performed to evaluate the expression. In these experiments, about 30 embryos from microinjected eggs were tested each day for activity. The data in Figure 3 summarize the relative levels of expression during this 10-day period; the autoradiograph of the highest expression of each construct is shown in Figure 4. Consistent with expression in tissue cultured cells, the peak activities of CAT were highest with pFV-2, which were about 2–3 times higher than those from pSV2cat, pRSVcat, and pFV-1. pSV2cat was more active *in vivo* than *in vitro* while pHSPcat was inactive *in vivo* with or without heat shock treatment.

Although the maximal levels of expression in developing zebrafish in the 10-day period were generally similar to the levels of expression in tissue cultured cells, the period of active expression was dramatically different for the four vectors during the 10-day course of development. As shown in Figure 3, pFV-2 gave maximal expression three days after microinjection when all other constructs were inactive. This high level of expression continued for three days, and then dropped after the fifth day. In contrast, pSV2cat and pFV-1 were active only for a single day, the fifth day after microinjection. Similarly, pRSVcat showed its highest expression only on the sixth day after microinjection. Supercoiled DNAs persist in microinjected embryos for at least two weeks (data not shown), indicating that the decrease in CAT activity is due to some form of gene regulation. We are not aware of any specific event that occurs during this interval of development.

DISCUSSION

In this study, we constructed vectors with the CAT reporter gene driven by promoter elements of piscine origin and linked to the chinook salmon poly(A) signal. The poly(A) signal of the carp β-actin gene, which contains a regulatory sequence that suppresses expression in muscle cells[24], could be used in future vectors. The complex promoter of the carp β-actin gene[15] enabled us to make different expression vectors with varying potentials for expression. Expression was investigated with regard to the strength and duration of promoter activity in transfected cells and in microinjected embryos. We have demonstrated that the pFV-2 vector containing the β-actin promoter/enhancer complex was 2–5 times more active than any other promoter tested. A vector with only the proximal promoter of the carp β-actin gene, pFV-1, directed comparable amount of CAT synthesis to that directed by the strong RSV and SV40 promoter/enhancer complexes. This result was expected since in all non-muscle vertebrate cells, β-actin is one of the most abundant mRNA species due to its strong promoter[25]. Since the transcriptional regulatory sequences for the β-actin gene are conserved throughout all vertebrates examined to date[26], our fish expression vectors should be active in all species of fish.

The strong, constitutive RSV promoter was highly active in cultured vertebrate cells and in microinjected embryos. In contrast, the SV40 promoter was comparably active in mouse L cells and in zebrafish but less active in carp EPC cells. These results are consistent with previous observations that the SV40 promoter does not simply respond to constitutively-expressed transcriptional factors, rather it responds to factors expressed in certain cell

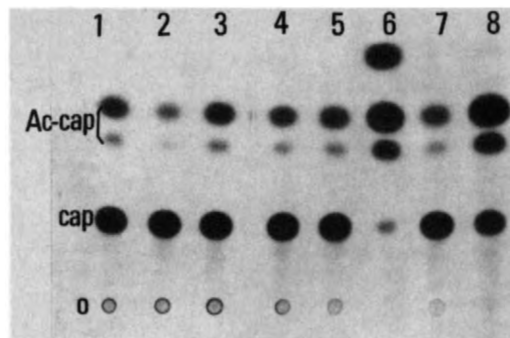

FIGURE 2 Test of expression vectors in mouse L cells by CAT analysis. Acetylated derivatives of ^{14}C-labelled chloramphenicol were produced during one hour incubations with equal amounts[36] of protein extracts from transfected mouse L cells, separated on thin layer plates, and visualized by autoradiography. After exposure to film, the spots were cut out of the plates and quantified by liquid scintillation counting. The average expression levels of CAT from each construct, from four separate determinations, are summarized in Table 1. (1) pRSVcat (2) pHSPcat without heat shock treatment (3), pHSPcat with heat shock treatment at 42°C for one hour three hours before the cells were harvested (4) pSV2cat (5) pFV-1 (6) pFV-2; (7) pFV-2 with 1/5 amount of cell extract used in the assay (8) 0.07 units of pure CAT enzyme from Sigma. (o) origin (cap) ^{14}C-chloramphenicol (Ac-cap) acetylated forms of ^{14}C-chloramphenicol.

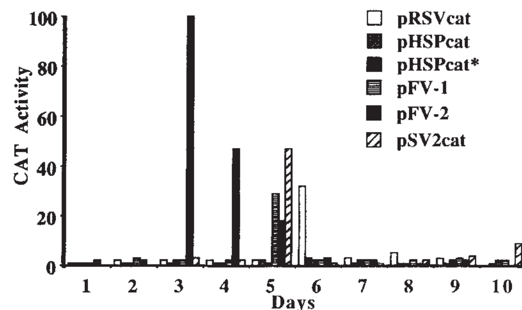

FIGURE 3 Time course analysis of expression vectors in zebrafish. Constructs were microinjected into fertilized eggs of zebrafish. Samples of 30 embryos or fingerlings were taken daily for 10 days for analysis after microinjection. All the values were normalized relative to that of pFV-2 on the third day after injection, the maximal expression. The CAT analysis was performed as described. Conventions are the same as in Figure 2. pHSPcat*, pHSPcat with heat shock treatment at 42°C for one hour three hours before analysis.

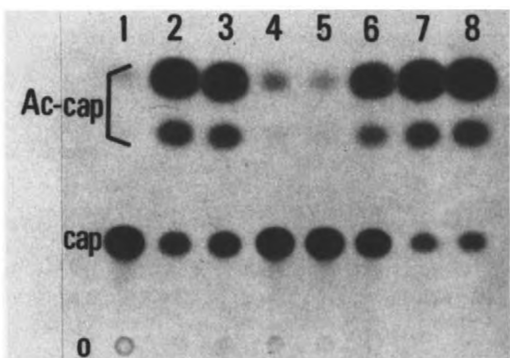

FIGURE 4 Maximal expression of the vectors in zebrafish. Conventions are the same as in Figure 3 with the maximal expression during the 10-day period presented. (1) pCAT-1 (2) pRSVcat (3) pSV2cat (4) pHSPcat without heat shock treatment (5) pHSPcat with heat shock treatment at 42C for one hour three hours before analysis (6) pFV-1 (7) pFV-2; (8) 0.07 units of pure CAT enzyme from Sigma. (o) orgin (cap) ^{14}C-chloramphenicol (Ac-cap) acetylated forms of ^{14}C-chloramphenicol.

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types or to factors induced during differentiation[25,27]. As reported by others[28], the human heat shock protein 70 promoter showed a 3–5 fold enhanced activity following heat shock induction in tissue culture but it was not tightly regulated since about 50-fold expression above background was evident without heat shock treatment. However this promoter was inactive in zebrafish following heat shock treatment. The lack of induction of the *hsp* promoter may be due to the inability of the heat shock regulatory proteins in zebrafish to recognize the human heat shock regulatory sequences[20,29,30]. Alternatively, the heat shock regime which was adequate for cultured fish cells was nonetheless insufficient to induce heat shock *in vivo*.

The expression patterns of all of our vectors in transgenic fish embryos are striking. The peak expression level of each construct occured only for a brief period from one to three days. The dramatic changes of expression from background to maximal level and then back to background did not correlate with any known developmental activity of the animal. The fish hatched from the embryo 2–2½ days after injection, reflecting nearly synchronous development (±6 hours) in all the eggs and hatchlings. We expected that maternal expression and developmental switches would occur around hatching. Before the expression peaks, the low expression may be explained by the possibility that the transcriptional factors required by these promoters were either not expressed or expressed at a low level. The rapid decay of expression is unexplained since β-actin genes should be highly expressed in nearly all cells during rapid cellular multiplication that accompanies rapid growth of the hatchling. We are examining causes of these patterns of expression in zebrafish to determine if plasmid instability or other factors may be influencing gene expression. We have found high expression of FV-1 and FV-2, containing the chinook salmon growth hormone gene, 10–20 days after injection into wallye and northern pike fish eggs where development is slower than in zebrafish,[19] (Moav et al., manuscript in preparation).

In conclusion, we anticipate that the highly expressible vectors with piscine promoter, intron, and poly(A) signal sequences will be useful in genetic engineering of fish. The CAT gene in our constructs can be, and has been, replaced by genes of research as well as economic interest to produce fish with the appropriate phenotypes.

EXPERIMENTAL PROTOCOL

Construction of CAT plasmids for promoter insertion. Construction of recombinant molecules, transformation into *E. coli*, and analysis of recombinant clones were performed according to established procedures[31,32]. Vectors used for cloning were pUC118 or pUC119[33] as specified and *E. coli* strain JM101[34] was used as bacterial host throughout. The 1.6 kb HindIII/BamHI fragment with the CAT gene and SV40 3′ sequence was purified by gel electrophoresis[35], filled-in by the Klenow fragment of *E. coli* DNA polymerase I. As shown in Figure 1A, pCAT-1 was generated by use of SstI linkers and pCAT-2 by use of SalI linkers. To eliminate the SV40 sequences, pCAT-2 was digested with SalI and XhoII, which cut the DNA at original BamHI/BglII junction of the CAT gene and the SV40 sequences. The SalI/XhoII fragment containing the CAT gene was cloned into the SalI/BamHI sites of pUC119 and the resulting plasmid was designated pCAT-3; note that the BamHI site was regenerated by this recombination. pCAT-3 was digested with SalI, filled-in by the Klenow fragment polymerase, ligated to KpnI linkers, and recircularized. The resulting plasmid was designated pCAT-4. For construction of pFV-1, the SalI/BamHI fragment of pCAT-3 was cloned into the SalI/BamHI sites of pRC6sGH[15]. The BamHI site was created by digesting the plasmid with XbaI (located behind the termination codon of the salmon growth hormone gene but ahead of the poly(A) signal of the growth hormone mRNA), filling-in by Klenow polymerase, and ligating to BamHI linkers. For the pHSPCAT construct, we used the human *hsp*70 promoter, located in a BamHI/HindIII fragment on plasmid p17. p17 was first digested with BamHI, filled-in by Klenow polymerase, ligated to HindIII linkers, and then digested with HindIII. The resulting fragment was cloned into the HindIII site of pUC119, generating pHSP. The Chinook salmon growth hormone cDNA clone was obtained from Choi Hew (Toronto, Canada), PRSVcat and pSV2cat were obtained from Bruce Howard (NIH). p17 was obtained from Richard Voellmy (U. Florida).

Cell lines and their cultivation. Mouse L fibroblast (TK^-) cells and carp epithelial EPC cells were grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum (Gibbco) supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 25 U/ml of nystatin. The mouse L cells were grown at 37°C and the EPC cells at 28°C, both supplied with constant 5% CO_2.

DNA transfection. Expression plasmids were introduced into cultured mouse cells by DEAE-dextran method[21] and into EPC cells by calcium phosphate precipitation method[22] with modification[23]. The EPC cells were sensitive to DEAE-dextran as well as glycerol and DMSO shock. Consequently the transfection efficiencies using the traditional calcium phosphate precipitation method were low. Fish cells were treated with trypsin prior to transfection. The resulting cell suspension was incubated in the calcium phosphate/DNA precipitate. This procedure increased the surface area of the cells in contact with the DNA added for transfer thereby elevating the transfection efficiency.

Microinjection. Aided by a dissecting scope and a Leitz micromanipulator, microinjection was carried out in a petri dish containing 10% Hanks solution in well water. A glass microneedle with an inner diameter of 3 μm at the tip was drawn with a Leitz needle-drawing apparatus. After loading the DNA solution into the micro-needle, the micromanipulator was carefully adjusted to insert the tip of the micro-needle into the central position of the germinal disc of the fertilized egg. About 30,000 molecules of DNA were then released into each egg by using a proper pressure. The microinjected eggs were incubated in 10% Hanks solution in well water at 28°C until analysis. Random samples of 30 embryos were taken each day after microinjection for analysis.

Cell extract preparation and CAT assay. Cell extracts were prepared and CAT assays were performed according to Lotapa et al.[21] with cultured cells. Developing embryos or fingerlings were homogenized with a Polytron homogenizer (Brinkmann Instruments Co.) for 10 sec. The homogenates were then frozen in a dry ice/ethanol bath for 5 min., and thawed in a 37°C water bath for five min. The freezing/thawing lysis procedure was repeated 3 times. The lysates were then centrifuged in a microfuge for two min. The pellets were discarded and the supernatants were collected for CAT analysis. Protein amounts were quantified by the Bradford method[36]. An equal amount of protein (about 50 μg) was used for CAT assays. CAT activity was analyzed by thin layer chromatography on silicon gel plates (J. T. Baker). After autoradiography, the spots were cut out of the gel plates for quantification by scintillation counting.

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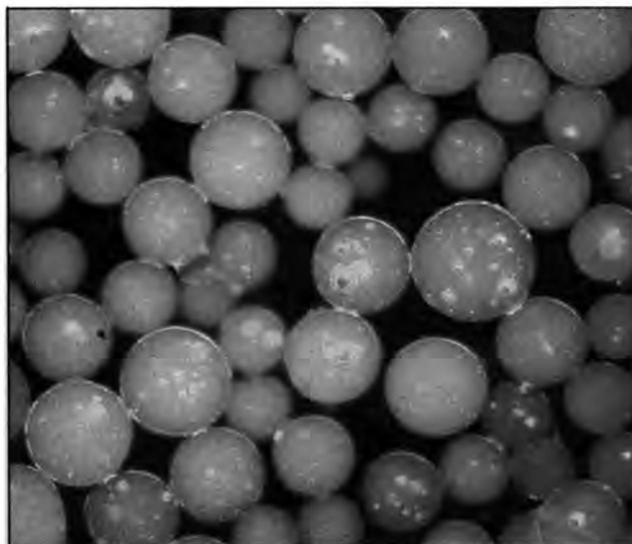

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