Quantitative Analysis of the Accumulation of Zein mRNA during Maize Endosperm Development*

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In order to characterize the heterogeneity and expression of maize zein genes, we constructed and characterized a cDNA library of endosperm mRNAs. Clones from the library that were of sufficient size to be full-length or near full-length copies of zein mRNA were characterized by restriction enzyme mapping and cross-hybridization analysis. Based on these comparisons we found three classes of zein sequences corresponding to proteins of M, 22,000, five corresponding to proteins of M, 19,000, and a single one corresponding to a protein of M, 15,000. Representative clones from these nine groups were used as probes to measure levels of the corresponding mRNAs in developing endosperms. It was found that these groups represent varying amounts of transcripts that range from 2 to 20% of the total endosperm mRNA population. For the M, 19,000 and M, 22,000 zein clones there is a correlation between the amount of mRNAs and the apparent number of genes in the genome. The relative level of mRNA for the M, 15,000 zein was found to be 3 times that of the M, 22,000 and M, 19,000 zeins, suggesting that these genes are transcribed at a higher rate during endosperm development or that their mRNAs are more stable.

Zeins, the storage proteins of the maize seed, are composed of a group of alcohol-soluble proteins. These proteins are synthesized in the endosperm between 12 and 50 days after pollination (DAP), and they account for 50–60% of the total seed protein (Wilson, 1983). Zeins are resolved into five molecular weight classes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The four smaller molecular weight classes (M, 22,000, 19,000, 15,000, and 10,000) are similar in amino acid composition, while a protein of M, 27,000, referred to as the reduced soluble protein (Wilson, 1983), is distinguishable from the others by its solubility in buffered saline. The M, 22,000 and 19,000 zeins can be resolved into several differently charged polypeptides by two-dimensional gel electrophoresis (Hurstman et al., 1981). An analysis of zeins from the maize inbred W64A revealed five species of M, 22,000 zeins and four to six species of M, 19,000 zeins. The M, 15,000 and 10,000 zeins, as well as the reduced soluble protein, were observed as single spots on two-dimensional gels.

The mRNAs encoding zeins are translated on membrane-bound ribosomes (Larkins et al., 1978; Burr et al., 1978). The zein polypeptides appear to be transported into the lumen of the rough endoplasmic reticulum, where they aggregate to form protein bodies that are 1–2 μm in diameter (Kho and Wolf, 1970; Larkins et al., 1978).

Genes encoding zeins have been mapped at distantly spaced loci on chromosomes 4, 7, and 10 (Soave et al., 1978; Valenti et al., 1979; Salamini and Soave, 1982). Estimates for zein gene number range from as few as 20 (Pedersen et al., 1980) to as many as 150 (Viotti et al., 1979). Recently, Wilson and Larkins (1984) used cloned zein cDNAs in Southern blot hybridization and estimated that there are approximately 50 M, 19,000 zein genes, 25 M, 22,000 zein genes, and only 1–2 M, 15,000 zein genes in the maize inbred W64A.

Many mutations affect zein synthesis. Perhaps the best characterized is the opaque-2 mutation (Nelson, 1969). In this mutant, zein synthesis begins several days later and ceases earlier than in the normal genotype (Jones et al., 1977). This results in a 50% reduction in zein content in the mutant relative to the normal genotype. Although all zein polypeptides are substantially reduced, the M, 22,000 proteins are nearly absent (Jones et al., 1977; Pedersen et al., 1980).

Patterns of zein gene expression have not been thoroughly examined. Because the zein genes occur in different linkage groups and on several chromosomes, there may be differences in the timing of transcription and level of expression of these genes. To better characterize the timing of zein polypeptide synthesis, we have isolated these proteins from protein bodies at 12, 16, and 22 DAP and resolved them on two-dimensional gels. In addition, zein proteins from an opaque-2 mutant were analyzed. We have also characterized the accumulation of specific zein mRNAs in normal and opaque-2 endosperms by using cDNA clones in a sensitive dot hybridization assay.

MATERIALS AND METHODS

RESULTS

As a first step to examine the developmental expression of zein genes, we analyzed the accumulation of the zein proteins in the maize inbreds W64A and W64A opaque-2. Proteins were extracted from protein bodies isolated at 12, 16, and 22 DAP and subjected to two-dimensional polyacrylamide electrophoresis. In the normal inbred, many of the M, 22,000 and 19,000 zeins were detectable by 12 DAP (Fig. 1). By 16 DAP several additional M, 22,000 and 19,000 zeins were observed.
and the $M_r$, 15,000 polypeptide was present in more significant amounts (Fig. 1B). Interestingly, the $M_r$, 27,000 polypeptide or reduced-soluble protein was not synthesized until 16 DAP. After 16 DAP there were no qualitative changes in the pattern of polypeptides present in protein bodies of the normal genotype.

In the opaque-2 inbred, significant amounts of zeins were not detectable in the endosperm until after 12 DAP (Fig. 1, D–F). At all stages examined the $M_r$, 22,000 polypeptides were present in very small amounts relative to the normal genotype. But as was true of the normal genotype, there were no detectable qualitative differences in the synthesis of zein proteins after early stages of endosperm formation.

Construction of Zein cDNA Clones—To assess the complexity of zein mRNA, we constructed and characterized full-length zein cDNA clones. In a previous report we analyzed zein cDNAs that were synthesized using size-fractionated endosperm poly(A) RNA from 18 DAP membrane-bound polysomes (Marks and Larkins, 1982). Several of the recombinant plasmids contained cDNA inserts of sufficient size to

**Fig. 1.** Analysis of zein proteins by two-dimensional gel electrophoresis. Proteins were extracted from protein bodies with 70% ethanol plus 1% 2-mercaptoethanol and resolved by two-dimensional gel electrophoresis. The nonequilibrium pH gradient from left to right was between 9.1 and 4.2. The apparent molecular weights are indicated at the right. The protein bodies were isolated from normal endosperms at (A) 12 DAP, (B) 16 DAP, (C) 22 DAP, and from opaque-2 endosperms at (D) 12 DAP, (E) 16 DAP, and (F) 22 DAP.
represent full-length copies of zein mRNAs (900–1000 bases). However, most contained short cDNA inserts (average of 400 base pairs). We were able to isolate a larger proportion of long cDNA clones by incorporating two changes into our cloning protocol. First, double-stranded cDNAs were size fractionated on 5% acrylamide slab gels instead of sucrose gradients. Approximately 1–2% of the double-stranded cDNA was of the same size as zein mRNA. Secondly, by using the pUC 8-JM83 transformation system (Messing and Vieira, 1982), we obtained a 10-fold increase in cloning efficiency over the pBR 322-HB101 system that was previously used.

More than 60 recombinant plasmids were analyzed that contained cDNA inserts over 800 nucleotides in length. These clones were subgrouped based on their restriction enzyme patterns (Fig. 2) and their ability to cross-hybridize to zein mRNAs (900-1000 bases). We were able to isolate a larger proportion of long clones that were previously characterized (Marks and Larkins, 1982). Several clones that did not cross-hybridize to previously characterized zein sequences were shown to correspond to zein mRNAs by DNA sequencing. Four of the 60 clones contained cDNA inserts over 800 nucleotides in length. These clones were subgrouped based on their restriction enzyme patterns and represented rare endosperm mRNAs as determined by a quantitative dot hybridization assay (data not shown).

Cross-hybridization Analysis of Zein Clones—Clones representing closely related zein mRNAs can be distinguished in cross-hybridization analyses by using stringent conditions. In a previous analysis we demonstrated that the two closely related zein clones cZ22A-1 and cZ22B-1, which are 94% homologous, can be distinguished from one another at $T_m = -15 \, ^\circ C$ (Marks and Larkins, 1982). By using these conditions we were able to identify nine distinct groups of zein sequences (Fig. 3). Eight of these groups, Z19 A through D, Z22 A through C, and Z15 A, do not cross-hybridize at $T_m = -15 \, ^\circ C$. However, the ninth group, Z19 AB, does cross-hybridize to the Z19A and Z19 B groups.

Zein mRNA Quantification—It was previously shown that zein mRNAs begin to accumulate in the endosperm at 12 DAP and reach maximum levels between 18 and 22 DAP (Marks et al., 1983). Thereafter, the levels of mRNAs decline somewhat until seed maturation. To determine if there is temporal regulation of gene expression and if groups of genes are differentially expressed, we quantified the levels of mRNA corresponding to different clone groups among poly(A) RNAs of both normal and opaque-2 genotypes at 12, 18, and 28 DAP. This was done with a dot hybridization assay in which replica filters were spotted with triplicate DNA from representative zein plasmids and an ovalbumin clone. Each filter was hybridized to 35 ng of $^{32}P$-labeled cDNA that was synthesized using endosperm poly(A) RNA as a template (Fig. 4). To standardize the assay a series of filters was hybridized to 35 ng of DNA that was synthesized with 18 DAP mRNA and 0.5, 2.5, and 12.5 ng of ovalbumin cDNA. The amount of labeled cDNA that annealed to the ovalbumin DNA was determined directly by liquid scintillation counting, and this was used to generate a standard curve of cpm annealed versus ng of ovalbumin cDNA. The amount of cDNA corresponding to each of the M, 19,000 and 22,000 zein clone groups was estimated based on this curve. Because the mRNA encoding the M, 15,000 zein has a higher GC content than that of ovalbumin, a correction factor was introduced to obtain the
Analysis of Zein mRNA Accumulation

FIG. 4. Quantification of zein mRNA accumulation during endosperm development. Filters A through F were spotted with the cloned zein sequences indicated along the left side. Filters were hybridized to 35 ng of cDNA synthesized using poly(A) mRNA isolated from: A, 12 DAP W64A endosperm; B, 18 DAP W64A endosperm; C, 28 DAP W64A endosperm; D, 12 DAP W64A opaque-2 endosperm; E, 18 DAP W64A opaque-2 endosperm; and F, 28 DAP W64A opaque-2 endosperm. The hybridization conditions and cDNA synthesis are described under “Materials and Methods.”

In the normal genotype, mRNA was detected at 12 DAP for eight of the nine zein groups (Fig. 5A). The remaining group corresponded to a rare class of zein mRNA. This mRNA may have been present at 12 DAP at a concentration below the limit of detection, which was 0.5% of the total poly(A) RNA for this assay. At 12 DAP 16% of the endosperm poly(A) RNA was homologous to the zein clones and by 18 DAP this percentage increased to 75%. The amount of mRNA which corresponded to each group ranged from 2% (Z19D) to 20% (Z19C) of the endosperm poly(A) RNA by 18 DAP. Most of the groups represented 6–12% of the endosperm poly(A) RNA. Based on this analysis, the maximum levels of mRNA were maintained until 28 DAP. We did not examine the fate of the individual groups of mRNAs after this time; however, in previous studies it was shown that after 28 DAP the zein mRNA levels decline gradually (Marks et al., 1983).

In the opaque-2 mutant, zein mRNAs were not detected until after 12 DAP (Fig. 5B). By 18 DAP the mRNA for the M, 19,000 and 15,000 zeins accounted for 12% of the endosperm poly(A) RNA. The mRNAs corresponding to the M, 22,000 zeins could not be detected by this assay at any stage during endosperm development. At 28 DAP the zein mRNAs corresponding to the M, 19,000 and 15,000 groups accounted for 32% of the total cDNA. Strikingly, the mRNA for the M, 15,000 protein did not increase during this period. The maximum levels of mRNA that corresponded to specific zein groups in opaque-2 ranged from 1% (Z19D) to 15% (Z19C).
These values are lower than those obtained for the normal genotype. Previous analyses showed that zein mRNA levels in opaque-2 reached a maximum at 22 DAP and declined dramatically after 28 DAP (Marks et al., 1983).

**DISCUSSION**

To characterize the developmental regulation of maize zein genes, we analyzed qualitative changes in zein proteins of protein bodies from developing endosperms of normal and opaque-2 genotypes. We also investigated qualitative and quantitative changes in zein mRNA populations during endosperm development. In other systems where proteins are encoded by multigene families, evidence has been found for changes in the pattern of gene expression during development (McKeown and Firtel, 1981; Sim et al., 1979; Weintraub et al., 1981). Although we found no evidence for zein gene switching during endosperm development, we did observe differences in the timing of expression during early stages of seed formation. Several of the M, 22,000 and 19,000 polypeptides were detected in protein bodies before others, and their synthesis preceded that of the M, 27,000 polypeptide (RS protein) and the M, 15,000 polypeptide. But after 16 DAP, we could not distinguish any significant changes in the pattern of polypeptides present in protein bodies.

To obtain a more quantitative analysis of zein gene expression, we utilized a sensitive dot hybridization assay that allowed us to compare the accumulation of specific groups of mRNAs during endosperm development. In this assay representative cDNA clones were immobilized on nitrocellulose filters, and the filters were allowed to hybridize to 32P-labeled cDNAs synthesized from poly(A) RNAs of developmentally staged endosperms. Under certain hybridization conditions, quantitative distinctions between related sequences can be made (Beltz et al., 1983). For such studies a hybridization criterion must be determined that will prevent moderately related sequences from cross-hybridizing, while allowing closely related sequences to hybridize in a reasonable length of time. The amount of DNA on the nitrocellulose filter must be in excess over the homologous probe sequence. The length of the filter-bound DNA sequence must fully represent that of the corresponding cDNAs in the probe population or the rate of hybridization will be influenced by the length of the clones. Finally, the specific activity of all the probes must be the same, or at least known, so separate hybridization reactions can be compared. For the results obtained in the present study, all of these requirements were fulfilled. Although there was some variation in the results of replications of the hybridization reactions, the values obtained appeared to be quantitative. For example, the hybridization of the c19A and c19B groups was roughly equivalent to that of the c19AB group (Fig. 5A).

The developmental expression of nine distinct groups of zein mRNAs that corresponded to the M, 22,000, 19,000, and 15,000 zeins was analyzed. We were unable to identify clones corresponding to the M, 27,000 and 10,000 polypeptides in the cDNA library. In the W64A genotype zein mRNAs were detected in endosperms by 12 DAP for eight of the nine groups analyzed. By this early stage of development mRNAs encoding the M, 19,000 polypeptides accounted for 25–30% of their maximum level, whereas mRNAs encoding the M, 15,000 and 22,000 polypeptides represented only 10% of their maximum. Assuming the mRNAs have similar stabilities, this suggests that transcription of the genes encoding the M, 19,000 polypeptides is initiated earlier or is more active than that of the genes encoding the M, 22,000 and 19,000 zeins.

In the opaque-2 mutant, transcripts corresponding to the M, 19,000 and M, 15,000 zeins were not detected in the endosperm until after 12 DAP and then only at reduced levels. Transcripts for the M, 22,000 zein groups remained below detectable limits at all the times tested. This result is in agreement with earlier studies that demonstrated a drastic reduction in mRNAs encoding the M, 22,000 polypeptides (Pedersen et al., 1980; Marks et al., 1983).

Wilson and Larkins (1984) estimated that there are 24 Z19 (A + B + AB) genes, 30 Z19C genes, 7 Z22A genes, 8 Z22B genes, and 2 Z15A genes. In a separate analysis it was shown that there are 2 Z19D genes (Marks et al., 1985). It is unlikely that all these genes are functional, since a number of pseudogenes have been identified (Spens et al., 1983; Kridl et al., 1984). However, if we assume that there is not a significantly large number of pseudogenes we can estimate the relative transcriptional activity for the M, 19,000 and 22,000 zein genes. At 18 DAP each gene in a clone group or subfamily accounts for approximately 1% of the mRNA (Table I). There is some variation in the percentage of mRNA/gene, and this may be due to a number of factors. Some gene groups may contain more pseudogenes than others, or there may be differences in the stability of the mRNAs. Part of the variation may reflect differences in gene transcription.

The gene(s) encoding M, 15,000 zeins appear to be more actively transcribed than those encoding M, 19,000 and 22,000 zeins. It was estimated that there are two genes encoding the M, 15,000 polypeptide in the W64A, yet at 18 DAP the M, 15,000 zein mRNAs account for 6.5% of the endosperm mRNA (Table I). If neither of the genes is a pseudogene then each is responsible for about 3% of the mRNA at 18 DAP. This is significantly more mRNA/gene than was estimated for the M, 19,000 and 22,000 zein genes. The higher level of expression may reflect more active transcription or differences in the mRNA stability. It is unlikely to be due to differences of efficiency in reverse transcription, since high levels of the mRNA were also apparent in Northern blot hybridizations of endosperm mRNA.

**REFERENCES**

Beltz, G. A., Jacobs, K. A., Eickbush, T. H., Chertas, P. T., and Kafatos, F. C. (1983) *Methods Enzymol.* 100, 266–284.

Buell, G. N., Wickens, M. P., Payvar, F., and Schmick, R. T. (1978) *J. Biol. Chem.* 253, 2471–2482.

Burr, B., Burr, F. A., Rubenstein, I., and Simon, M. N. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 696–700.

Cesay, J., and Davison, N. (1973) *Nucletic Acids Res.* 5, 1539–1552.

Davison, N., Haebel, P., and Smithies, O. (1984) *Nucletic Acids Res.* 12, 587–595.

Grunstein, M., and Hoggness, D. S. (1975) *Proct. Natl. Acad. Sci. U. S. A.* 72, 3961–3965.

Horkmaa, W. J., Smith, L. D., Richter, J., and Larkins, B. A. (1981) *J. Cell Biol.* 89, 292–299.

Jones, R. A., Larkins, B. A., and Tsai, C. Y. (1977) *Plant Physiol.* 59, 625–629.
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Kafatos, F. C., Jones, D. W., and Efstratiadis, A. (1979) Nucleic Acids Res. 7, 1541–1552
Khoo, U., and Wolf, M. J. (1970) Am. J. Bot. 57, 1042–1050
Kridl, J. C., Vieira, J., Rubenstein, I., and Messing, J. (1984) Gene 28, 113–118
Marks, B. A., and Hurkman, W. J. (1978) Plant Physiol. 62, 256–263
Larkins, B. A., Pearlmutter, N. L., and Hurkman, W. J. (1978) in The Plant Seed: Development, Preservation, and Germination (Rubenstein, I., Phillips, R., Green, C., and Bengenbach, E., eds) pp. 49–65, Academic Press, New York
Maniatis, T., Fritche, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, p. 117, Cold Spring Harbor Laboratory, New York
Marks, M. D., and Larkins, B. A. (1982) J. Biol. Chem. 257, 9976–9985
Marks, M. D., Pedersen, K., Wilson, D. R., and Larkins, B. A. (1983) in Advances in Gene Technology: Molecular Genetics of Plants and Animals (Downey, K., Voellmy, R. W., Ahmad, F., and Schultz, J., eds) pp. 369–381, Academic Press, New York
Marks, M. D., Lindell, J. S., and Larkins, B. A. (1985) J. Biol. Chem. 260, 16451–16459
McDonnell, M. W., Simon, M. N., and Studier, F. W. (1977) J. Mol. Biol. 110, 119
McKeown, M., and Firtel, R. A. (1981) Cell 24, 799–807
Messing, J., and Vieira, J. (1982) Gene 29, 269–276
Morrison, D. A. (1979) Methods Enzymol. 65, 499–560
Nelson, O. E. (1969) Adv. Agron. 21, 171–194
O’Farrell, P. Z., Goodman, H. M., and O’Farrell, P. H. (1979) Cell 12, 1133–1142
Pedersen, K., Bloom, K. S., Anderson, J. N., Glover, D. V., and Larkins, B. A. (1980) Biochemistry 19, 1644–1650
Roychoudhury, R., Jay, E., and Wu, R. (1976) Nucleic Acids Res. 3, 101–116
Salamin, F., and Soave, C. (1982) in Maize for Biological Research (Sherman, W. F., ed) pp. 155–160, Plant Molecular Biology Assn., Charlottesville, VA
Sharp, P. A., Berk, A. J., and Berget, S. M. (1980) Methods Enzymol. 65, 750–768
Sim, G. K., Kafatos, F. C., Jones, C. W., Kochler, M. D., Efstratiadis, A., and Maniatis, T. (1979) Cell 19, 1385–1386
Soave, C., Sumun, N., Viotti, A., and Salamin, F. (1979) Theor. Appl. Genet. 52, 263–267
Spina, A., Viotti, A., and Pirrotta, V. (1983) J. Mol. Biol. 169, 799–811
Valentini, G., Soave, C., and Ottaviano, E. (1979) Heredit 42, 33–40
Viotti, A., Sala, E., Marotta, R., Alberi, P., Balducci, C., and Soave, C. (1979) Eur. J. Biochem. 102, 211–222
Weintraub, H., Larsen, A., and Groudine, M. (1981) Cell 24, 333–343
Wilson, M. C. (1983) in Seed Proteins: Biochemistry, Genetics, Nutritive Value (Gottschalk, W., and Muller, H. P., eds) pp. 271–307, Martinus Nijhoff/Junk, The Hague, Netherlands
Wilson, D. R., and Larkins, B. A. (1984) J. Mol. Evol. 20, 330–340
Woo, S. L. C., Beattie, W. G., Cattell, J. F., Dugnicyzyk, A., Staden, R., Brownlee, G. G., and O’Malley B. W. (1981) Biochemistry 20, 6457–6466

Supplement to: Quantitative analysis of the Accumulation of Zein mRNA during Endosperm Development, N. David Marks, Judith S. Lihlith, Brian A. Larkins

Materials and Methods

DNA restriction endonucleases, 32-P polymers, cloning fragment, and the dot blot apparatus were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Nucleic acid synthesis was obtained from Life Science, Inc. (St. Petersburg, FL) and polynucleotide transferase was from P-L Biochemicals (Milwaukee, WI). [α-32P]dCTP was purchased from New England Nuclear (Boston MA) or from Amersham (Chicago, IL). Nucleosides were obtained from Schleicher and Schuell (Keene, NH).

Two-dimensional Electrophoresis of Zein Polypeptides

Zeins were extracted from protein bodies with 70% ethanol, pH 13.2-14.0 as previously described (Hackett et al., 1981). For the first dimension, 130–150 µg of protein were resolved on non-equilibrium, isoelectric focusing gel (O’Farrell et al., 1977). The final pH of the gel was 5.4 at the top and 9.1 at the bottom. The gel was equilibrated in 50 mM Tris, pH 7.5, 10% glycerol, 2 M dithiothreitol, 0.01% SDS, 0.2% sodium dodecyl sulfate, and then at the top of 1.5% polyacrylamide gel. Electrophoresis was at 25 mA until a bromphenol blue front reached the bottom of the gel. The gel was stained in 0.1% comassie blue and destained in 15% methanol, 10% acetic acid.

Cloning of double-stranded cDNA

Zein mRNAs were isolated from membrane-bound polyribosomes of the maize hybrid W64A as described by Larkins and Burke (1978) and used as template for double-stranded (ds) cDNA synthesis as described by Helft et al. (1978). 32-P labeled ds-Zeins were fractionated on a 15% polyacrylamide gel. Gel slices that contained 500 or more bases were placed in dialysis bags filled with 1 ml of 0.3 M Tris (pH 7.4, 0.01 M EDTA, 0.3 M NaCl, 0.01 M mercaptobenzyl acid, 0.01 mM EDTA) and were subjected to 30 min elution of 300 nmoles of mRNA was recovered by ethanol precipitation in the presence of 0.6 M Na acetate. Nonpolymer tails of oligo (dC) were added to the 24 µm cDNA with DNA polymerase as described by Roychoudhury et al. (1977) and the ds cDNA was annealed to single-stranded DNA that was the complete in the method of Morrison (1979). Bacteria containing recombinant plasmides were identified by a modification of the method of Kunkel and Bogomolov (1977) using [32P]labeled cDNA as the probe.

Analysis of Sequence Homology among Zein cDNA Clones by Restriction Enzyme Analysis

Restriction enzyme maps of the cDNA clones were determined by analyzing the length of single or double digestion by gel electrophoresis. In cases where the complete DNA sequence was unknown, maps were generated by computer analysis (Severson, et al., 1984).

Cross-hybridization analysis was performed by the method of Kunkel and Bogomolov (1977). Bacteriophage DNA was isolated and purified according to Larkins and Burke (1982). Plasmids and prokaryotic cDNA were labeled with [32P]dCTP using a kit supplied by St. John Associates (Cambridge, MA). DNA was separated by gel electrophoresis and transferred to nitrocellulose paper as described by Maniatis (1983). The hybridization criteria were defined by Maniatis (1983), including the following: probe = 2 x 106 cpm on 0.6 x 105 probe (Sharp et al., 1988; Casey and Davidson, 1977).