4. Discussion

The two enzymatic activities "alkaline phosphatase" and "5' nucleotidase" have received considerable attention by laboratories working with *Dictyostelium* (Armant *et al.*, 1981, Bhanot *et* al, 1985, Loomis *et al.,* 1984 and Mohan Das *et al.,* 1984). The alkaline phosphatase was assayed with the artificial substrate *p*-nitrophenol phosphate, while the 5' nucleotidase was assayed with 5'AMP. It was not clear, however, if the two activities resided in a single protein. Alkaline phosphatase and 5' Nucleotidase from various sources have been studied and found out that they are designed under one name although they differ in their physical and kinetic characters (Fleit *et al.,* 1975, Fritzson, 1967, Schwabe *et al.,* 1975 and Phillis *et al.,* 1976). Furthermore in *Dictyostelium discoideum*, according to MacLeod and Loomis (1979), 5NU and AP were the product from a single gene locus. Also they found that the only one form of the enzyme appears throughout the development of *Dictyostelium.* Also, Lee *et al.* (1975); Gezelius and Wright (1965) predicted that alkaline phosphatase in *Dictyostelium* may actually be a 5NU. Armant and Rutherford (1981) found the copurification of both activities in a homogeneous preparation of the protein.

4.1. Purification of 5NU and AP

In order to better understand the role of this enzyme(s), I purified the proteins again according to Armant *et al.,* 1981. Culminant cells were found to provide higher activity of the enzyme than amoeba AX3K cells of *Dictyostelium.* This indicated that the enzyme was developmentally regulated. It was possible that the enzyme is produced at all stages of development of *Dictyostelium,* and that an inhibitor is present at the early stage of development. During development, the inhibitor becomes inactive or removed, so the enzyme can become active. In fact, Mohan Das and Weeks (1980, 1981) presented evidence for the existence of such an inhibitor of alkaline phosphatase activity. Because the 5NU is a membrane bound protein (integral membrane protein), the interaction of the protein with the lipid portion of membranes is stabilized by the predominantly hydrophobic nature of their surface (Spatz and Stritmatter, 1971). Triton X-100 was very important for the purification of this protein because Triton X-100 is a non-ionic detergent which can bind to the hydrophobic region of the proteins. Then, it can enhance the ability of the protein to solubilize in the buffer or a solution prepared in H_2O. Fernley *et al.* (1971) suggested that the 5NU and AP should not be lipoprotein because after lipid extraction of the proteins, the hydrophobic character and the activity of the proteins still remains. Also they suggested that its hydrophobicity should come from the abundance of non-polar surface residue of amino acids or glycosides.

Concanavalin A affinity was used to purify the 5NU and AP because of its glycoside properties. During this step of purification, the flow through was collected and the activity of the unbound protein was determined. A considerable proportion of the total activity was found in this flow-through fraction. It is unclear whether this represented a second form of the alkaline phosphatase or is enzyme that lost the glucoside linkage during the step of solubilization with Triton X-100.

DEAE HPLC and SW300 gel filtration chromatography were used to purify the 5NU and AP also. Each step of the purification was assayed by the degradation of *p*-

nitrophenyl phosphate. The result still indicated that only one form of the enzymes was present. In order to separate a subunit or a copurifying protein, the active fractions were separated by SDS PAGE and stained. Three bands of 90, 120 and >120 kDa were always found. The polypeptide at a molecular weight of 90 kDa was found to degrade both *p*-Nitrophenyl phosphate, and Nitro Blue Tetrazolium-5-Bromo-4-Chloro-3-Indolyl Phosphate. This result coincided with the estimation of the molecular weight of the protein as determined by SW300 gel filtration. This value differed from the 120 kDa as reported by Armant and Rutherford (1981). The difference could be due to the strain of *Dictyostelium* were used as starting material. Armant and Rutherford (1981) used *Dictyostelium discoideum* strain NC-4 while I used strain AX3K to purify the 5NU and AP.

A major obstacle was encountered in attempts to obtain enough protein for sequencing. Triton X-100 using for solubilizing the membrane bound protein was found to cause a smear on denaturing gels. Biobeads were used to overcome this problem; a ratio of 1:10 Triton X-100 and biobeads was required. The protein was sequenced from the Edmund degradation method and by Mass Spectrometry, but only 3 small fragments were obtained.

4.2. Developmental regulation of *5NU* as determined by RT-PCR

In order to obtain the full length of the *5NU,* degenerate primers were designed from the available partial sequences. cDNA from amoeba and 20 h of development were used as a template. When phos N and phos B5 were used as 5' and 3' primer, a product of PCR amplification was obtained from slug cDNA but not from amoeba cDNA. It can be concluded that the cDNA was synthesized from the message of *5NU* because the first strand cDNA was synthesized using phos B5 as the 3' primer. This was my first indication that the *5NU* gene was developmentally regulated. In order to determine that cDNA was synthesized, rather than amplification of genomic sequences, various marker genes were used. All marker genes were amplified using primers that surrounded an intron. The presence or absence of this intron indicates that the PCR product was derived from amplification of DNA or RNA/cDNA, respectively. Glycogen synthase is included as an additional constutively expressed gene, even though it is expressed at low levels throughout the life cycle of *Dictyostelium* (Williamson *et al.,* 1996). This makes it a good marker gene for the presence of rare messages in the synthesized cDNA. The *gp-2* gene is inactive during vegetative growth, but becomes transcriptionally active very early in development during aggregation (Rutherford *et al.,* 1992). In addition to its use in determining the precision of the time points collected, *gp-2* also served as a good marker gene for the presence of long messages (the expected size of *gp-2* cDNA is around 3.2 kb). *PDI* generates the smallest message (0.95 kb) and is expressed form the onset of starvation to about 8 h of development (Frank *et al.,* 1991). This gene serves as a temporal marker for the precision of the time points, as well as a marker for the presence of smaller messages.

4.3. Separate genes encode *5NU* and "classical" *AP*

When the protein sequence obtained for the purified 5NU was used to search the *Dictyostelium* developmental cDNA project (University of Tsukuba, Japan), several

clones of the same gene were present that matched the *5NU* peptides. I also searched this database from the cDNA project with conserved sequences of "classical" alkaline phosphatases from other organisms. This search revealed several clones of the same gene, none of which corresponded to the purified *5NU* gene. Partial sequences of both genes from the 3' end of the cDNA were known. I then obtained the whole cDNA sequences of both genes. The results showed that both genes contained different sequences. There was no overlapping sequences between the two genes.

A cDNA sequence from both genes was used as a probe for Southern blot analysis. A single form of both *5NU* and *AP* was found. The restriction enzymes that used to digest high molecular weight genomic DNA were selected from the restriction sites as determined from the DNA sequence. High temperature of hybridization and high stringency washes insured that the hybridized bands were specific. When degenerate oligonucleotides from the protein sequencing by Edmund degradation and Mass Spectrometry were used in Southern Analysis, a similar pattern of hybridized bands was observed confirming that the 5NU protein is the product from one single gene. Considering the cDNA sequences of both genes, the 1,921 bp cDNA of *5NU* should produce a protein of molecular weight 70 kDa while the 1,827 bp cDNA of *AP* should produce a protein of molecular weight 67 kDa. Thus the proteins are of a similar size so they can not be separated clearly on a denaturing gel, as described before.

4.4. The expression of the *5NU* and *AP* during development of *Dictyostelium*

Both genes were studied for their expression during development. RNA extraction from each stage of development were shown to result in undegraded products, as shown by visible 17s and 26s rRNA bands on the formaldehyde gel after staining with Ethidium bromide. The intensity of 26s rRNA band was twice that of the 17s rRNA band as expected. Also, the equal amount of RNA from each stage was compared to the intensity of both rRNA bands from each lane on the gel. *Actin 8* was also used for Northern blot analysis to determine the condition of RNA from each stage of development. *Actin 8* was found to be constitutively expressed at constant, high levels throughout the life cycle of *Dictyostelium,* in accordance with the results of Romans *et al.,* 1985. The *actin* probe was the PCR product from two primers designed to encompass the start codon of the gene. The positive result from Northern blot analysis indicated that the full length of *actin* gene was isolated. Also we replied heavily on the diversity of various mRNAs in the isolated RNA from each stage of development. Several probes were used to determine the quality of the mRNA. The results showed that the RNA from one isolation contained *gp-2, actin, TFII, AP* and *5NU.* Using these RNA preparations, different patterns of expression of *AP* and *5NU* was found. The *5NU* is developmentally regulated while the *AP* is expressed at all stages of development in *Dictyostelium.* There was no expression of *5NU* before 4.5 h. It may be the result of de novo mRNA synthesis or, alternatively differential degradation of the message. More experiments will be performed to determine the mechanism of this expression of the *5NU.* The exact size of both messages was not determined, but were estimated to by slightly smaller than 2 kb as compared to the size of the 17s (1,900 bp) and 26s (4,100 bp) rRNA bands on the exposed film. This coincides with the length of sequenced cDNA of *AP* and *5NU* (1,827 and 1,921 bp, respectively).

4.5. Similarity of 5NU to a protein involved in cell-cell interaction during development in *Dictyostelium*

Although no close similarity existed between the 5NU protein and any other protein in databank searches, it is perhaps significant that the protein with closest similarity was a *Dictyostelium* protein called Contact Site A (Siu, Gerish). Contact Site A and 5NU showed 24% identity over 151 amino acids. This cell adhesion molecule (MW 80,000) is expressed at the aggregation stage, between 6 and 10 h of development, and is known to be under cAMP regulation. It is known to mediate the EDTA-resistant type of cell-cell adhesion via homophilic interaction. The protein is preferentially associated with cell surface filopodia, which are probably involved in the initiation of contact formation between cells.

4.6. Attempts at gene disruption of the *5NU* and *AP* genes

A "knockout" vector for both *5NU* and *AP* was constructed using a *blasticidin* resistant gene flanked by an *actin 8* terminator and an *actin 15* promoter. A directional construct was obtained from the *AP* digested with *Nsi* and *Mun*I and interrupted with the BSR cassette digested with *Eco*RI and *Pst*I. In addition, a non-directional construct was obtained if the *5NU* and BSR cassette which both were digested with *Bam*HI and then ligated. Because the length of BSR cassette is long, about 1,376 bp, the difference of cDNA with or without BSR interruption could be detected easily by PCR amplification using two primers designed to encompass the restriction site (*Bam*HI in the *5NU; Nsi* and *Mun*I in the *AP*). Optimum conditions for each pair of primers was determined for annealing temperature and concentration of $MgCl_2$. The BSR cassette flanked by 5' and 3' cDNA sequence was used to transform and integrate into the wild type genome of *Dictyostelium.* Due to the unusual base composition of *Dictyostelium* (78% A/T overall, although 90% A/T are in non-coding regions), there is an increased rate of instability for some cloned fragments (Nullen, 1987). With respect to the sequence of *5NU,* the upstream sequence contains repeated sequences of A and T. This could be an explanation for non-specific binding, as these repeated sequences are common in most *Dictyostelium* genes. Further attempts at knockout mutagenesis will use constructs in which these repeated sequences are deleted.

The second possibility for non-specific integration could be that I transformed the linearized BSR cassette (flanked by 5' and 3' *5NU* sequence) together with the linearized pBlueScript vector; both of them share compatible ends. After transformation into *Dictyostelium* genome, the two DNA fragments may have been religated to from intact plasmid. This can interfere with the homologous recombination (Danny Fuller, University of California at San Diego, personal communication). I attempted to prevent religation by treating the ends of both DNA fragments with calf intestinal alkaline phosphatase to remove phosphate group at 5' sequence. However, it has been shown that *in vivo* ligation can occur whether the ends introduced are sticky, blunt, or even incompatible (Shah-Mahoney *et al.*, 1997). Further experiments will use additional methods to interfere with end-to-end joining of transfecting molecules.

The third possibility for our inability to detect homologous recombination is that the disruption of the *5NU* in *Dictyostelium* is lethal. In this case, after gene replacement, the transformed cells die immediately. Only the non-specific recombinants will survive when treated with blasticidin. The lethality of the disruption would seem unlikely because the *5NU* gene shows little on no expression in undifferentiated amoeba cells. There remains the possibility that a low level of mRNA and protein expression does occur in amoeba, and that this protein is required for normal cell growth.

5. Summary

The results reported in this dissertation clarify some aspects of the role of alkaline phosphatase and 5' nucleotidase in *Dictyostelium,* and, in addition, raise other questions. I have purified the 5NU protein, obtained protein sequence, used this sequence to obtain a cDNA clone, and have shown this gene is expressed only during cell differentiation. In addition I have described a *Dictyostelium* clone that codes for "classical" alkaline phosphatase. These sequences of these two genes are obviously different, and, therefore, it can be concluded that 5NU and AP are different proteins. This result may resolve some of the confusion in the *Dictyostelium* literature over these proteins, for different laboratories may have been studying two different gene products. Clearly both 5NU and AP are able to degrade the artificial substrate pNPP. Likewise, both of the proteins prefer an alkaline pH for optimum activity. Therefore, 5NU is an alkaline phosphatase, but it is not the "alkaline phosphatase" generally referred to by that name in other organisms. Because both clones are now available, it opens the way for analysis of the *in vivo* function of these proteins.

It remains to be determined why the size of the protein derived from the *5NU* cDNA differs from that estimated by SDS PAGE. As determined in the result section, Triton X-100 interfered with the migration of the polypeptide in SDS gels, and this may have resulted in an incorrect estimation of the size of the protein. However gel filtration chromatographic analysis of the protein also predicted a molecular weight larger than that derived for the cDNA sequence. It is also possible that a portion of the cDNA sequence has been deleted during cloning. Others in our laboratory are attempting to obtain a genomic clone of the *5NU*, and the sequencing of this clone should answer some of these questions.

I was not able to target the *5NU* gene for knockout mutagenesis with the current construct. It is possible that the *5NU* gene product is required for cell maintenance and, therefore, mutations in the gene are lethal. Although knockout mutagenesis in *Dictyostelium* has been used several times in the past, success with the technique is not always predictable. With some genes one construct will fail, while another will result clear replacement of the endogenous gene. Future attempts at knockout mutagenesis of *5NU* will utilize different regions of the genes. If these experiments are successful for both *5NU* and *AP,* the role of these two proteins will undoubtedly become more clear.

It is also of interest that a search of the database with the *5NU* sequence resulted in *Contact Site A* of *Dictyostelium* as the protein with closest similarity. As discussed previously, this membrane-bound glycoprotein is involved in cell to cell interactions during cell differentiation of *Dictyostelium.* Does the similarity of the *5NU* and *Contact Site A* sequences indicate a function of *5NU* in cell-cell communication, rather that a role in degradation of extracellular 5'AMP, as I originally proposed? Or is the phosphatase activity somehow a part of the cell-cell interactions that occur during the differentiation of the two cell types in *Dictyostelium*?

Although much need to be accomplished to define the role of *5NU* in cell differentiation, the facts that are now available only enhance the interest in this gene. We now know that the *5NU* gene is expressed only in differentiating cells. The alkaline phosphatase that is active in amoeba is a different gene product. In addition it was known previously that the 5NU protein takes a position between the two developing cell types with its phosphatase catalytic site facing extracellularly. We now know the 5NU contains sequences similar a protein involved in cell-cell interactions. Thus, it is likely that understanding the role of this protein in cell differentiation will continue to receive the attention of researchers interested in this area of research.

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