A novel component of the ubiquitination system, called NOSA, is essential for cellular differentiation in Dictyostelium discoideum. Disruption of nosA does not affect the growth rate but causes an arrest in development after the cells have aggregated. nosA contains seven exons and codes for a developmentally regulated 3.5-kb mRNA. The 125-kDa NOSA protein is present in the cytosol at constant levels during growth and development. The C-terminal region of NOSA has homology with ubiquitin fusion degradation protein-2 (UFD2) of Saccharomyces cerevisiae and putative homologs in Caenorhabditis elegans and humans. UFD2 is involved in the ubiquitin-mediated degradation of model substrates in which ubiquitin forms part of the translation product, but ufd2 mutants have no detectable phenotype. In accord with the homology to UFD2, we found differences in the ubiquitination patterns between nosA mutants and their parental cell line. While general in vivo and in vitro ubiquitination is minimally affected, ubiquitination of individual proteins is altered throughout growth and development in nosA mutants. These findings suggest that events involving ubiquitination are critical for progression through the aggregate stage of the Dictyostelium life cycle.

Protein levels and activities are regulated by differential gene expression, translational regulation, and post-translational modification. One such modification is the attachment of the small 8-kDa ubiquitin protein to a specific set of substrates, which targets these proteins to proteasomes for degradation. Although the ubiquitin pathway has the capacity to degrade almost every protein, it is a benign component of the cytoplasm and the nucleus. The protein degradation cascade begins when ubiquitin is attached to ubiquitin-activating enzymes of the E1 class through a high energy thioester bond. Activated ubiquitin is then transferred to substrates by ubiquitin-conjugating enzymes of the E2 class. The carboxyl-terminal glycine of ubiquitin forms a covalent bond with the ε-NH₂ group of a lysine in the target protein. In some cases, the conjugation of ubiquitin to proteins also requires the E3 ubiquitin ligases, which form complexes with specific E2 conjugating enzymes and the substrate, to confer specificity. Subsequent cycles result in the formation of multicmeric ubiquitin chains on the target protein that are recognized by the 19 S cap of the proteasome. The multiubiquitin chain is removed by ubiquitin-specific hydrolases before the protein is unfolded and enters the 20 S proteasome for degradation (see Ref. 1 for a review).

Ubiquitin-mediated proteolysis serves diverse cellular functions. In addition to cell cycle regulation by degradation of cyclins (for reviews, see Refs. 2 and 3), it has been implicated in generation of free amino acids (4), removal of dysfunctional proteins (5), major histocompatibility complex antigen presentation (6), regulation of the inflammatory response (7), degradation of regulatory proteins that control cell growth (8, 9), and regulation of cellular differentiation (10–12).

Developmental regulation by proteolytic pathways is also critical to the morphogenesis of the soil amoeba D. discoideum. This organism has a variety of motile and developmental behaviors; in addition to forming cysts, the organism is capable of aggregation and subsequent differentiation into stalk and spore cells, as shown in Fig. 1A. Developmental mutants defective in various components of the ubiquitin pathway have been isolated. The mutations have been mapped to the genes encoding the proteasomal subunit PRTC (13), the deubiquitinating enzyme UBPA,2 and the conjugating enzyme UBC1 (14). In a genetic screen to identify genes essential for cellular differentiation, we isolated a gene we have called nosA (for no spores). The disruption of nosA results in a developmental arrest at the tight aggregate stage; occasional aggregates go on to form fruiting bodies that lack spores. The developmental blockade is stage-specific, despite the fact that the protein is present throughout growth and development. The carboxyl-terminal 525 amino acids of NOSA share 57% similarity with the UFD2 protein of Saccharomyces cerevisiae. UFD2 is a component of the ubiquitin fusion degradation pathway, which has been studied in S. cerevisiae for its ability to degrade ubiquitin-β-galactosidase fusion proteins (15). Mutagenic analysis of the lysine residues in the ubiquitin domain of a ubiquitin-β-galactosidase test protein suggests that the internal lysines at positions 29 and 48 of ubiquitin are the major attachment sites for additional ubiquitins. The ubiquitination at Lys⁴⁸ requires UFD2. A physiological role for UFD2 has not been established, because the S. cerevisiae mutant has no apparent phenotype other than the failure to degrade the test substrate, and no
natural substrate has been identified.

To investigate whether NOSA is involved in ubiquitination during development of Dictyostelium, we examined the distribution of ubiquitin conjugates. The disruption of nosA had no effect on the stability of the endogenous ubiquitin fusion protein Ubex52, which resembles the model substrates used for studying UFD2 in S. cerevisiae. However, we found altered patterns of ubiquitination in dividing and developing nosA mutant cells. We identified at least three proteins that are stabilized in the nosA mutant, suggesting that the role of the NOSA protein during development is to remove a specific set of proteins to ensure developmental progression. The isolation of nosA in D. discoideum links a component of the ubiquitin fusion degradation pathway to cellular differentiation and defines a distinctive phenotype that can be employed for further biochemical and genetic studies.

**Experimental Procedures**

**Strains**—The original mutation was created in a variant of AX3 called DH1, which is a uracil auxotroph (16). The strain MYC1 was derived from AX3 by disruption of the pyr5-6 locus using pMYC10 as a marker and was named by Peter Devreotes.

**Development**—For multicellular development, axenically growing cells in midlog phase (2 × 10^6 cells/ml) were washed twice in SorC buffer (16.7 mM Na₃H₂PO₄, 50 mM CaCl₂ (pH 6.0)). The cells were resuspended in SorC buffer and plated on nitrocellulose filters (Millipore Corp.), which rested on SorC-saturated Whatman filter pads (grade 17), at a density of 8.1 × 10^5 cells/cm².

**Restriction Enzyme-mediated Integration (REMI)**—The strain DH1 was electroporated in the presence of the restriction enzyme described (17). DH1 and MYC1 were provided by Peter Devreotes.

**Gene Recovery**—The genomic fragment containing nosA was isolated by taking advantage of the selectable plasmid pJB1, which contains pBluescript, inserted into exon VII (Fig. 4A). Genomic DNA from the nosA mutant was isolated, cut with a number of restriction enzymes, religated, and transformed into the E. coli strain DH15a. In this way, the genomic fragments flanked by BclI (1.9-kb) and BstXI (3.4 kb), and HindII (3.1 kb) sites were isolated. For the recovery of the 5’ end of nosA, genomic DNA from the parental strain DH1 was cut with BstBI, size-fractionated by agarose gel electrophoresis, and eluted from the gel. Fragments in the range of 3–4 kb were ligated with pBluescript II KS (Stratagene) that had been digested with Clal and dephosphorylated. The ligation mixture was used to transform E. coli strain DH10b. The resulting plasmid library was screened with the 5’-P-labeled 0.55-kb PstI fragment of nosA.

**Isolement d’un Partial cDNA**—Total RNA was prepared as described (20) from axenically grown cells in log phase (about 5 × 10^6 cells/ml) that were starved for 6 h in SorC at 2 × 10^5/ml. From this preparation, poly(A)^+ RNA was purified by using the PolyATtract mRNA isolation system (Promega). Double-stranded cDNA was generated from 0.9 μg of poly(A)^+ RNA using the Marathon cDNA amplification kit (Clontech). The nosA cDNA was prepared by reverse transcription PCR, employing primer pairs that define two overlapping fragments of nosA. Toq polymerase (Promega) was used in all PCR reactions, unless otherwise indicated. The 5’ portion of the nosA gene was amplified using the primers nos-4 (5’-AAAGTATACCAATGATGAAG-3’), nos-6 (5’-AAGCTTCTGAGGATGCCTTAAA-3’), and nos-8 (5’-AACCTTCTGAGGATGCCTTAAA-3’). The 3’-end of nosA was amplified using the primers nos-2 (5’-AAATTGATACCAATGATGAAG-3’), nos-4, and nos-6. The amplified PCR product was digested with BglII and XhoI and subcloned into pGEM-T Easy (+) (Promega). The resulting plasmid was linearized with SacI and XhoI, and the SacI/XhoI fragment from pGEM-TOPO was inserted into plasmid pcDNA3/SX1. This plasmid was digested with SacI and XhoI and the nosA cDNA fragment of 2.54 kb was inserted into the expression vector pDXA-HC (21).

The transcriptional start site was mapped by using the 5’-rapid amplification of cDNA ends system, version 2 (Life Technologies, Inc.). The genomic cDNA was first linearized with SacI, RNA was isolated using the primer EXT-3 (5’-ATTGGGGTCTTCAACTG-3’). The original RNA template was removed by RNase H treatment, and the first strand DNA was purified with a spin column (Qiagen). After 45 cycles of the 3’-end of the cDNA, the 5’-extension product was amplified using the provided anchor primer and EXT-4 (5’-AAGCAGGCACGTGTTTCCCTT-3’). The amplified product was subcloned into the TA cloning vector pCR2.1 (Invitrogen) for sequencing. The complete nosA cDNA sequence was derived from the partial cDNA and the 5’-extension.

**Preparation of Antibodies**—A polyclonal antibody against NOSA was generated by Eurogentec (Seraing, Belgium). A synthetic peptide comprising the C-terminal amino acids ETKKKIDELWASKKQ of NOSA was prepared and conjugated to keyhole limpet hemocyanin (Sigma) according to the method described (26). The conjugated peptide was injected into rabbits (New Zealand White), boosted twice, and then tested on crude cell extracts from Dictyostelium strain DH1 and F11 by Western blot. Polyclonal antibodies against ubiquitin were from Sigma. Antiserum against a synthetic peptide corresponding to the carboxyl terminus of human UbCEP52 (Ubex52) were a gift from K. L. Redman and were prepared as described (27).

**Western Blots**—Dictyostelium cells harvested at various times during development on filters were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer. The proteins from 5 × 10^6 cells were size-fractionated on 60, 7.5, or 12.5% SDS-polyacrylamide gels. Immobilized proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp.) with a semidy electromobliter (Integrated Separation System) according to the manufacturer’s protocol. Equal loading was verified by staining with the 0.2% Coomassie brilliant blue R-250 in 50 mM H₃BO₃, 37% acetic acid. Blots were blocked with 5% nonfat dry milk in TBS (20 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.01% Merthiolate), prior to incubation with the primary antibody. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma). The immobilized proteins were detected on x-ray film by chemiluminescence with Luminol as the substrate (Renaissance, DuPont).

**Constructions**—The plasmid pJB1 carries a fragment of the D. discoideum genome containing pyr5–6 (28) in the Clal site of pBluescript II-KS (Stratagene). For the deletion of the nosA locus, two knockout constructs were made, p5’-Δ3’/bar and pNOSA-3’/bar (see Fig. 4 for location of restriction sites in the nosA locus). To construct p5’-Δ3’/bar, the blasticidin resistance (bar) cassette (29) from pBlR319 was subcloned into pBluescript as a PstI fragment. The 1.2-kb BglII/XhoI fragment of nosA, which had been previously subcloned into pBluescript, was released by restriction with Smal and SacI and was inserted downstream of the bar cassette. The 0.8-kb PstI/EcoRI fragment of nosA, which was previously subcloned into pUC19, was released by restriction with HindIII and EcoRI and inserted upstream of the bar cassette. The resulting plasmid, p5’-Δ3’/bar, was restricted with HindIII and SacI, and the 3.4-kb fragment containing the bar cassette flanked by the genomic nosA fragments was used for transformation of DH1 to generate the nosA mutant 2E4.

**Chimera Experiments**— nosA mutant cells (F11) were transformed with a plasmid that expresses β-galactosidase as described by Hadwiger and Firtel (30). Parental DH1 cells and transformed F11 cells were microinjected in a ratio of 9:1 and allowed to develop on nitrocellulose filters (see Fig. 6 for filtration). The cultures were fixed at various stages of development and stained for β-galactosidase activity as described (31).
13,000 × g at 4 °C, and the supernatant was saved. Ubiquitin (Sigma U6253) was purified by gel filtration on a 40-ml (4.5 cm) Sephadex G50 column equilibrated with Tris buffer. The purified ubiquitin was labeled with 125I (from NEN Life Science Products) using IODO-GEN column equilibrated with Tris buffer. The purified ubiquitin was separated from free iodine on a Sephadex G25 column equilibrated with Tris buffer. Mg-ATP contained 50 mM MgCl2, 20 mM Na-ATP (pH 7.5), 10 mM dithiothreitol. Ubiquitination reactions were performed initially as described (33). ATP was required, but an ATP-regenerating system was not necessary. The following protocol was used after optimization for 125I-labeled ubiquitin incorporation: 9 μl of extract (about 8 × 107 cells/ml); 9 μl of 125I-labeled ubiquitin (60 μCi/ml), 3 μl of Mg-ATP, 9 μl of 50 mM Tris-HCl (pH 7.4). Reactions were incubated for 0–60 min at 30 °C, and aliquots were loaded on SDS-polyacrylamide gels. The gels were stained with enhancing screens.

RESULTS

Generation of the nosA Mutant—The nosA mutant was isolated in a REMI mutagenesis screen to identify developmental genes (18). A population of uracil auxotrophs was mutagenized and selected for uracil prototrophy, and then 5000–10,000 transformants were examined individually as plaques growing on bacterial lawns. The colonies were inspected for morphological aberrations. The nosA mutant, initially called R7, was blocked at the tight aggregate stage, which corresponds to stage 9 in Fig. 1, A and E, all nosA mutants aggregate and arrest after about 12 h at what is called the tight aggregate stage (Fig. 1B). At this stage, all cells have aggregated and start to differentiate into prespore or prestalk cells. Sometimes aggregates succeed in making a stalk and even produce a spore (data not shown).

The Phenotype of nosA Mutants—Instead of forming fruiting bodies (stage 9 in Fig. 1, A and E), all nosA mutants aggregate and arrest after about 12 h at what is called the tight aggregate stage (Fig. 1B). At this stage, all cells have aggregated and start to differentiate into prespore or prestalk cells. Sometimes aggregates succeed in making a stalk and even produce a spore (data not shown).

Because the integration of pJB1 during REMI mutagenesis was at the 3'-end of the gene, we created the gene replacement mutant 2E4 (see “Experimental Procedures” and Fig. 4A), which had the nosA phenotype. Northern blots with a partial nosA cDNA as a probe showed that 2E4 is a nosA null mutant producing no transcript (data not shown).

In D. discoideum, a number of genes are expressed in one cell type but not in the other. These genes can be used to monitor the developmental stage reached by mutants. The extracellular matrix protein-coding gene, ecmB, is a prestalk cell marker, and the spore coat protein coding gene, psA, is a prespore-specific marker. In Northern blots with RNA from developing wild-type and mutant cells, the nosA mutation reduces tran-
The mutant amoebae were marked with a β-galactosidase expression cassette driven by a constitutive actin 15 promoter (34), the mutant cells could be identified in chimeras with isogenic parental cells. In these mixtures, aggregation proceeded normally, but at the tight aggregate stage, the mutant cells were excluded and left in the periphery (Fig. 3). No mutants cells synergized with wild-type cells to form spores, which indicates an extreme cell-autonomous phenotype. The nosA mutation causes a cell-autonomous defect. When the mutant amoebae were marked with a β-galactosidase expression cassette driven by a constitutive actin 15 promoter (34), the mutant cells could be identified in chimeras with isogenic parental cells. In these mixtures, aggregation proceeded normally, but at the tight aggregate stage, the mutant cells were excluded and left in the periphery (Fig. 3). No mutant cells synergized with wild-type cells to form spores, which indicates an extreme cell-autonomous phenotype. The nosA mutant by itself arrested exclusively at the tight aggregate stage in these experiments.

**Gene Structure**—The genomic fragment containing nosA was isolated by plasmid rescue and from a genomic mini-library (see “Experimental Procedures”). The nosA locus has been mapped to chromosome 6 (35, 36). The NOSA gene consists of seven exons as shown in Fig. 4A. The coding sequence is flanked by a 5′-untranslated region of 181 nucleotides and a short 3′-untranslated region of 42 nucleotides as determined by comparison with different cDNAs. The short AT-rich introns of about 80–120 bp, which are flanked by conserved splice sites, are typical for *Dictyostelium* (37). A translational start codon was located and has the consensus sequences typical for *Dictyostelium* genes (37). A single open reading frame of 3.27 kb was identified in the genomic DNA and cDNA utilizing this start site. The length of the open reading frame plus untranslated sequences corresponds to the size of the mRNA detected in Northern blots. There is a polyglutamine and a polyasparagine stretch at the 5′-end of nosA. These have been described in other *Dictyostelium* genes (38), but their origin and functional significance are not known. The NOSA polypeptide does not contain any previously characterized motifs (39, 40). No sequence homology was found with the known E1, E2, or E3 enzymes of the ubiquitin-proteasome pathway.

The NOSA gene is developmentally regulated as a 3.5-kb transcript (Fig. 5A). Expression is at its peak at 4 h, when the cells are in the initial stages of aggregation, remains high through aggregation, and then decreases. Transcript is present at all stages, including vegetative growth. Western blotting with a NOSA antibody revealed a protein of the expected 125-kDa size in wild-type cells present throughout growth and development (Fig. 5B). The fact that the antibody does not detect the NOSA protein in the nosA mutant shows that the antibody is specific for NOSA (Fig. 5C). The disturbance of in vitro ubiquitination patterns (see below) throughout growth and development follows the temporal expression of NOSA. Gel filtration revealed that NOSA behaved like a globular protein with a molecular weight of 125,000–200,000 and is not part of a large complex (data not shown). High speed sedimentation analysis (1 h at 170,000 × g) followed by Western blotting demonstrated that NOSA is a cytosolic protein (data not shown).

### Homology to UFD2

The first indication of a role for nosA in ubiquitin-mediated proteolysis came from BLAST homology searches (41) in which nosA was found to be related to the *S. cerevisiae* sequence ufd2. In *S. cerevisiae*, the ufd2 product is necessary for degradation of an artificial substrate in which ubiquitin is coded as part of the protein, instead of being added post-translationally. The C-terminal 525 amino acids of NOSA share 57% similarity with UFD2, which includes an internal region of 75–85% homology that we call the NOCT region (for NOSA-C-terminal region) as shown in Fig. 4.

**Ubiquitin Fusion Proteins in Dictyostelium**—The suggested role of the UFD pathway is the degradation of ubiquitin fusion proteins. Two such proteins occur naturally: Ubex52 and Ubex72 (42, 43). These genes code for ubiquitin plus 52- and 72-amino acid C-terminal extensions, respectively, which are found in ribosomes. They may resemble the artificial substrates used by Johnson et al. (15) to recover the ufd mutants, because ubiquitin forms a part of the translational product. Antibodies prepared against the 52-amino acid extension of Ubex52 (27) detected the *Dictyostelium* protein in Western blots. As shown in Fig. 6, the detected protein migrates well below the apparent molecular mass of 16-kDa for Ubex52 (44) and is not recognized by a polyclonal ubiquitin antibody (data not shown). This indicates that this protein represents the processed C-terminal extension of Ubex52, although others have detected predominantly the uncleaved form (45). In any event, we could not detect any difference in the levels of this protein in the wild-type and the nosA mutant, which excludes Ubex52 as a substrate for NOSA.

In *Dictyostelium*, ubiquitination pathways are induced by stress (46). We asked whether the nosA mutation had any effect on the heat shock response. Cells were heat-shocked at 30 °C for 30 and 60 min, and ubiquitin conjugates were studied by Western blot analysis with an antibody against ubiquitin. Wild-type and nosA mutant cells responded similarly to the heat shock by inducing the ubiquitination system: ubiquitin conjugates increase during heat shock, especially at higher molecular weights, and this increase is also observed in the absence of NOSA. There is no significant difference in the appearance of ubiquitin conjugates in wild-type and nosA mutant after exposure to heat shock (data not shown).

### Altered Patterns of Ubiquitin Conjugation

The homology to UFD2 suggests a role for NOSA in the ubiquitination system. We asked whether the ubiquitination pattern is altered in the nosA mutant due to the absence of NOSA. Western blots probed with a ubiquitin antibody were used to study the pattern of ubiquitin conjugates in wild-type and in the nosA mutant. As shown in Fig. 7A, we could not detect a major difference in the appearance of such conjugates. To test whether ubiquitination of specific substrates is affected by the nosA mutation, we applied an *in vitro* ubiquitination system. Cell extracts from
the wild type and the \( \text{nosA} \) mutants were incubated with \(^{125}\text{I}\)-labeled ubiquitin (Fig. 7B). We detected differences in the ubiquitination pattern between the parental DH1 strain and the \( \text{nosA} \) mutants F11 and 2E4. Preferential labeling of proteins of approximately 66 and 87 kDa is detected in the \( \text{nosA} \) mutants (Fig. 7B). When the same samples from DH1 and 2E4 were size-fractionated on higher resolution polyacrylamide gels, an additional ubiquitin conjugate of about 125 kDa is detected in the \( \text{nosA} \) mutant (Fig. 7C). The observation that the same proteins accumulate in independent \( \text{nosA} \) mutants indicates that this effect is due to the absence of NOSA. The differences are observed in extracts prepared from growing and starving cells. Although the effect of the \( \text{nosA} \) mutation is only manifest at the tight aggregate stage of development, the changes that the mutation evokes in protein ubiquitination are not developmentally regulated. This is consistent with the observation that the levels of NOSA do not change during development (Fig. 5B).

**DISCUSSION**

We isolated the NOSA gene in a genetic screen designed to identify genes that are essential for the cellular differentiation of \( D. \text{discoideum} \). The disruption of \( \text{nosA} \) causes developmental arrest at the tight aggregate stage, when cells start to differentiate into the two precursor cell types, prespore and prestalk cells. The \( \text{nosA} \) mutation cannot be complemented by the pres-
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Fig. 5. Expression pattern of nosA transcript and NOSA protein. A, Northern blot analysis on growing or developing amoebas. Five µg of total RNA from cells (MYC1 strain) harvested at the indicated time points was size-fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized to a random primer-labeled DNA probe corresponding to the 1.6-kb Bell/EcoRI fragment of the genomic sequence. The 3.5-kb nosA transcript is indicated by an arrow. Equal loading was confirmed by ethidium bromide staining of the ribosomal bands (not shown). B, Western blot analysis of developing DH1 cells. Cellular proteins from 5 x 10^5 DH1 cells, harvested at the indicated time points, were size-fractionated on a 6.0% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The Western blot was reacted with NOSA antiserum (1:600 dilution) and developed as described under "Experimental Procedures." The processed Ubex52 blot was reacted with Ubex52 antiserum (1:800 dilution) and developed as described under "Experimental Procedures." The NOSA protein (125 kDa) is indicated by the upper arrow. The lower arrow indicates a degradation product of NOSA. C, Western blot analysis of DH1 and the nosA mutant (F11). The numbers above the gel lanes indicate hours of development.

Fig. 6. The nosA mutation has no effect on the levels of ubiquitin fusion protein Ubex52. Levels of Ubex52 in DH1 and the nosA mutant (F11). Cellular proteins from 5 x 10^5 growing cells (0) and cells starved for 5 h (5) were size-fractionated on a 12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The Western blot was reacted with Ubex52 antiserum (1:800 dilution) and developed as described under "Experimental Procedures." The processed Ubex52 protein is indicated by an arrow.

The current work supports the idea that NOSA is involved in a novel branch of the ubiquitination and proteolysis mechanism. UFD2 from S. cerevisiae has been implicated in the ubiquitin-mediated degradation of ubiquitin-β-galactosidase fusion proteins. The C-terminal portion of NOSA includes a region of 75–85% similarity with the other UFD2 homologs in Caenorhabditis elegans and humans. This region, which we call the NOCT region, is shown in Fig. 4B. NOSA and its homologs do not share motifs with known enzymes of the ubiquitin-proteasome pathway, such as the E1, E2, or E3 enzymes, which suggests a novel role for this class of proteins that is conserved throughout evolution. This role remains to be identified, but centrifugation and gel filtration show that NOSA is cytosolic and not part of a large complex such as the proteasome.

Mutagenic analysis of the lysine residues of the model substrates used to isolate ufd2 suggests that ubiquitination at Lys^{29} and Lys^{48} in the ubiquitin domain is required for targeting of the model substrate to the proteasome (15). Fig. 8 presents the proposed role of UFD2 in S. cerevisiae. The multiubiquitination at Lys^{48} requires UFD2, but the precise biochemical role of this protein is not understood. Based on the apparently normal growth of the ufd2 mutant, it is clear that UFD2 is not part of the major mechanism that mediates ubiquitin conjugation at Lys^{48} during ubiquitin multichain assembly of most endogenous proteins, because this activity is essential for viability (47). The ufd2 mutation affects ubiquitination at Lys^{48} only in the model substrate and probably does not block monoubiquitination of this residue but rather blocks the further formation of multibuqniitin chains. Multibuqniitination at Lys^{29} requires the E2 enzymes UBC4 and UBC5, as well as the E3 enzyme UFD4. The disruption of either ubc4b or ufd4 prevents ubiquitination at Lys^{29} but not ubiquitination at Lys^{48}. The reverse is also true; the disruption of ufd2 affects multibuqniitination at Lys^{48} but not at Lys^{29}. Therefore, the two lysine residues in this ubiquitin-β-galactosidase fusion are ubiquitinated by independent mechanisms. The UFD2-mediated ubiquitination at Lys^{48} is not essential for the degradation of a ubiquitin fusion protein with a reporter other than β-galactosidase, namely dihydrofolate reductase (15). This leads us to believe that the role of UFD2 and its related sequences in D. discoideum, C. elegans, and humans may be more complex than expected.

In support of a role for NOSA in the ubiquitination system, we demonstrate that, although the impact of the nosA mutation on the general ubiquitination pattern is mild, ubiquitination of a specific set of substrates is affected. In an in vitro ubiquitination assay, several proteins that are present at low levels in the wild-type extracts are prominently labeled in the mutant (Fig. 7). We propose that these proteins require ubiquitination at multiple lysine residues, one of which employs the activity of NOSA. We suggest that in the wild-type, these substrates are properly ubiquitinated and targeted to the proteasome. This results in a rapid turnover rate, so that these proteins are present at low levels when extracts are made for in vitro conjugation with 125I-labeled ubiquitin. In the absence of NOSA, these proteins accumulate in vivo due to incomplete multiqui- nitination because only the NOSA-independent mechanism is functional. This is insufficient for effective targeting. The larger amount of substrate would then be more abundantly labeled in vivo, again by the NOSA-independent ubiquitination machinery. This model is consistent with what we know about the activity of UFD2, as shown in Fig. 8.

In the search for natural substrates of NOSA, we examined the possibility that the ribosomal ubiquitin fusion protein Ubex52 (45) could be regulated by NOSA. If this is true, we would expect elevated levels of Ubex52 in the nosA mutant due to a defect in degradation. As shown in Fig. 6, we could not detect a difference in the levels of the processed form of Ubex52 of wild-type and mutant. Processing of Ubex52, which removes the ubiquitin domain, makes it an unlikely substrate of NOSA. NOSA is also not required for the increase in overall ubiquitination that follows heat shock. The NOSA gene itself, unlike the genes that code for ubiquitin, is not induced by heat shock (data not shown).

cAMP-dependent protein kinase A is known for its function in the final stages of sper differentiation. The phenotype of mutants in which the activity of the cAMP-dependent protein

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**Note:** The text appears to include a series of figures and tables, which are not visible in the provided text. The content seems to be discussing the role of Ubex52 and NOSA in ubiquitination and proteolysis, particularly in the context of D. discoideum and C. elegans. The text highlights the differences in ubiquitination between wild-type and mutant strains, suggesting a novel role for NOSA in this process.
The role of NOSA may be to eliminate regulatory proteins, which would enable cells to proceed along a developmental pathway. Such substrates could be negative regulators that must be removed to allow further differentiation but are retained in the nosA mutant. An alternative hypothesis is that NOSA is involved in suppressing the cell division cycle at the onset of development. In Dictyostelium, growth and development are strictly separated, and cells cease dividing once they enter development. In the absence of NOSA, one or more cell cycle events may continue and become deleterious, such that the initial part of the developmental program is activated, leading to aggregation, but abrupt failure occurs immediately after this stage. This is consistent with the presence of the protein and its ubiquitinating activity in dividing cells (Fig. 7). To elucidate the role of NOSA, it is important to identify the substrates of NOSA and other components that act in the same pathway. Genetic suppressor analysis and the identification of the proteins that are stabilized in the nosA mutant should provide answers to these questions.

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