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Ty3 *GAG3* and *POL3* Genes Encode the Components of Intracellular Particles

LORI J. HANSEN,† DOUGLAS L. CHALKER, KATHRYN J. ORLINSKY, AND SUZANNE B. SANDMEYER*

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717

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Ty3 is a *Saccharomyces cerevisiae* retrotransposon that integrates near the transcription initiation sites of polymerase III-transcribed genes. It is distinct from the copialike Ty1 and Ty2 retrotransposons of *S. cerevisiae* in both the sequences of encoded proteins and gene order. It is a member of the gypsylike family of retrotransposons which resemble animal retroviruses. This study was undertaken to investigate the nucleocapsid particle of a transpositionally active gypsylike retrotransposon. Characterization of extracts from cells in which Ty3 expression was induced showed the presence of Ty3 nucleoprotein complexes, or viruslike particles, that migrated on linear sucrose gradients with a size of 156S. These particles are composed of Ty3 RNA, full-length, linear DNA, and proteins. In this study, antibodies raised against peptides predicted from the Ty3 sequence were used to identify Ty3-encoded proteins. These include the capsid (26 kDa), nucleocapsid (9 kDa), and reverse transcriptase (55 kDa) proteins. Ty3 integrase proteins of 61 and 58 kDa were identified previously (L. J. Hansen and S. B. Sandmeyer, J. Virol. 64:2599–2607, 1990). Reverse transcriptase activity associated with the particles was measured by using exogenous and endogenous primer-templates. Immunofluorescence studies of cells overexpressing Ty3 revealed cytoplasmic clusters of immunoreactive proteins. Transmission electron microscopy showed that Ty3 viruslike particles are about 50 nm in diameter. Thus, despite the unusual position specificity of Ty3 upstream of tRNA-coding regions, aspects of the Ty3 life cycle are fundamentally similar to those of retroviruses.

The position-specific yeast retrotransposon Ty3 occurs in one to four copies in standard haploid laboratory strains of *Saccharomyces cerevisiae* (9). Ty3 is one of four retrotransposon families which occur in this yeast (reviewed in reference 3). Retrotransposons are similar to retroviruses in organization, replication, and integration. They differ from retroviruses in that they do not have an obligatory extracellular life cycle phase (reviewed in reference 1), and, as is the case for some defective endogenous retroviruses, they do not have an identifiable *env* gene. Ty3 is 5.4 kbp in length and consists of an internal domain flanked by long terminal repeats (LTRs) of the 340-bp sigma element (9). The nucleotide sequence of Ty3 (21, 22) showed that it is a member of the gypsylike class of retrotransposons (2). Ty3 contains two long open reading frames (ORFs), previously designated *TYA3* (307 codons) and *TYB3* (1270 codons), that were predicted to be analogous to the retroviral *gag* and *pol* genes, respectively (21). *TYA3* and *TYB3* will be referred to hereafter as *GAG3* and *POL3*, respectively, in order to reduce the potential for confusion with *TYA* and *TYB*, the first and second ORFs of Ty1 (17, 24, 47). Ty3 is unusual in that it integrates close to the transcription initiation site of polymerase III-transcribed genes (6, 7).

The *gag* gene of retroviruses encodes the structural proteins of the viral core, including the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins (reviewed in references 10, 12, and 46). An additional structural protein(s) and, in some retroviruses, protease (PR) can also be encoded by this gene. The protein predicted from the nucleotide sequence of *GAG3* has a proline-rich amino-terminal region, contains 14% basic residues, has a portion of the conserved CA motif (48), and has one copy of the C-X_2-C-X_4-H-X_4-C motif found in NC of retroviruses (reviewed in reference 12).

The retroviral *pol* gene encodes PR (for many retroviruses), reverse transcriptase (RT)/RNase H (RH), and integrase (IN). These products are expressed via a translational read-through mechanism as a *gag-pol* fusion protein. PR is required for processing of the fusion protein, RT is required for conversion of the RNA genome to DNA, and IN is required for integration of the DNA into the host genome. Ty3 *POL3*-encoded proteins are expressed as a *GAG3-POL3* fusion probably resulting from a +1 translational frameshift within a region in which the *GAG3* and *POL3* ORFs overlap (16). The *POL3*-encoded portion of the polyprotein contains domains which are homologous to retroviral PR, RT/RH, and IN sequences (20). The nucleotide sequences of two Ty3 elements, Ty3-1 and Ty3-2, have been determined and compared (21, 22). Although the sequence of Ty3-2 differs at only a small number of positions from the sequence of Ty3-1, Ty3-1 is transpositionally active and Ty3-2 is not. This difference was previously used, together with immunoblot analysis, to identify two protein species, of 61 and 58 kDa, at least one of which is required for integration.

Ty3 transposition is increased in cells that contain high levels of Ty3-1 transcripts (6, 21). We hypothesized that expression of these transcripts results in formation of nucleoprotein complexes, or viruslike particles (VLPs), such as those observed for Ty1 (18, 35, 37) and copia (14, 36, 41, 50). The properties of such a complex, if it existed, were of interest because of the integration specificity of Ty3. In this study, it was determined that cells which overexpress Ty3 do form nucleoprotein complexes. Major protein and nucleic acid species from the Ty3 VLP were identified, and com-

* Corresponding author.
† Present address: Department of Microbiology and Immunology, University of California, San Francisco, CA 94143.

plexes were localized within cells by immunofluorescence and electron microscopy.

MATERIALS AND METHODS

VLP preparation. Yeast strains were cultured by standard methodology (40). *S. cerevisiae* yVB110 (*MATa trp1-Δ901 gal3 ura3-52 his3-Δ200 ade2-101 lys2-1 leu1-12 can1-100* ΔTy*3*), a yeast strain containing no endogenous copies of Ty3 (21), was used for experiments in which Ty3 RNA, DNA, and proteins were identified. Fractionated cell extracts from an *spt3* strain, AGY9 (*MATa ura3-52 his4-539 lys2-801 trp1-Δ63 leu2-Δ1 spt3*) (a gift from A. Gabriel and J. Boeke, The Johns Hopkins University), were used for protein analysis and RT assays. The absence of the *SPT3* gene product in these strains results in reduced levels of full-length Ty1 transcripts (49). Yeast strains were transformed (27) with the high-copy-number plasmid, pEGTy3-1 (21), which contains the *GAL1-10* upstream activating sequence (28) fused to the Ty3-1 promoter. Transformed cells were grown in uracil-deficient synthetic medium to select for cells which retained the plasmid, while cultures of the parental strain were grown on uracil-containing synthetic medium. Both media contained 1% raffinose, a nonrepressing carbon source. Raffinose was replaced with 2% galactose for the induction experiments described here. Cultures were grown in liquid medium to an optical density at 600 nm (OD_{600}) of 0.9 to 1.1. Preparation of cell extracts and sucrose step gradient fractionation of induced and control cells were done essentially by the method of Eichinger and Boeke (13), as previously described (22); in some experiments, sucrose gradients contained 10 mM EDTA instead of 5 mM. Extracts representing 0.5 to 1 liter of induced cultures were fractionated through 30-ml 70/30/20% sucrose step gradients of 5, 5, and 20 ml, respectively. Four milliliters was reserved from the 70/30% interface per gradient. These fractions were concentrated by centrifugation in a Ti50 rotor (Beckman Instruments, Inc.) at 100,000 × *g* for 1 h at 4°C; for some analyses, this material was further fractionated over a 38-ml linear 15/50% sucrose gradient.

For determination of the S value of the Ty3 VLP, extracts from strain AGY9 were fractionated in step gradients and concentrated as described above. VLPs were further sedimented over an 11-ml linear 10/30% sucrose gradient, and aliquots of these gradients were loaded onto sodium dodecyl sulfate (SDS)–12% polyacrylamide gels and electrophoresed (see below). Ty3 proteins were visualized by silver staining, using a kit purchased from Bio-Rad Laboratories. In addition, the A_{280} of each fraction was determined by using an LKB Ultrospec II spectrophotometer. Migration of Ty3 VLPs on the sucrose gradient was compared with the migration of [^{35}S]methionine-labeled poliovirus (150S) and poliovirus procapsid (80S) (2a) (a gift from W. Blair and B. Semler, University of California, Irvine) run on a parallel gradient. Aliquots of 20 μl from each fraction were counted in an LS-230 scintillation counter (Beckman). Aliquots of the marker gradients were also fractionated on SDS–12% polyacrylamide gels to confirm the nature of the radioactive peaks. These gels were treated with Amplify (Amersham) and dried onto filter paper. The proteins were visualized by autoradiography (38).

Analysis of VLP proteins by SDS-polyacrylamide gel electrophoresis. Proteins from the 70/30% interface of the step gradient were concentrated by centrifugation. Pellets from the Ti50 centrifugation (representing 1 liter of the original culture) were suspended in 140 μl of buffer B (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.8], 15 mM KCl, 5 mM $MgCl_2$, 15% glycerol). Approximately 5 μg of total protein, as determined by Bradford assay (5), from each sample was mixed with 1/4 volume of 5× Laemmli sample buffer (31), boiled for 2 min, fractionated by electrophoresis on a SDS–10% polyacrylamide gel, and visualized by staining with Coomassie blue. The apparent molecular masses of the Ty3 proteins were determined by their mobilities relative to those of protein molecular weight standards (Bethesda Research Laboratories; Bio-Rad). For analysis of proteins from linear gradients, 80-μl aliquots from each 1.3-ml fraction were mixed with 20 μl of 5× Laemmli sample buffer, separated by electrophoresis in SDS–10% polyacrylamide gels, and visualized by silver staining (Bio-Rad).

Nucleic acid analysis. Fractions collected from both step and linear sucrose gradients were assayed for Ty3 RNA and DNA. Nucleic acids were recovered by adding an equal volume of 0.6 M sodium acetate (pH 7.0) together with 1 μg of *Escherichia coli* carrier tRNA to gradient fractions, followed by repeated extraction with phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were then precipitated at −20°C with 2.5 volumes of ethanol, collected by centrifugation, and dissolved in 60 μl water or 10 mM Tris (pH 8).

For the Northern (RNA) blot analysis, 10-μl aliquots were treated with DNase I at a concentration of 10 μg/ml in a solution of 50 mM Tris (pH 7.5) and 2 mM $MgCl_2$ at 37°C for 1 h. RNA was precipated with ethanol, redissolved in water, denatured by reaction with glyoxal at 50°C (34), fractionated by electrophoresis on a 1.1% agarose gel in 10 mM sodium phosphate buffer (pH 7.0), and blotted as described by Thomas (44). For the Southern blot analysis, 10-μl aliquots were treated with RNase A (10 μg/ml) and incubated at 37°C for 1 to 2 h. Samples were treated with restriction enzymes where indicated and then ethanol precipitated. The DNA was fractionated by electrophoresis on a 0.9% agarose gel in Tris-borate buffer (2.5 mM EDTA, 45 mM borate, 133 mM Tris [pH 8.3]) and transferred to nitrocellulose by the method of Southern (42). Nucleic acids were visualized as described previously (9) by hybridization to radiolabeled restriction fragments. The Ty3-specific probe was a 2,935-bp *Bgl*II fragment spanning positions 1130 to 4064 of the Ty3-1 internal domain. The sigma-specific probe was an *Eco*RI-*Hin*dIII restriction fragment from pSBS6 (9) containing an almost full-length sigma element.

RT assay. Ty3 RT activity was monitored by an exogenous primer-template assay modified from Garfinkel et al. (18). Strain AGY9, nontransformed or transformed with pEGTy3-1, was grown in synthetic medium containing galactose. Cell extracts were fractionated over sucrose step gradients as described above, and proteins from the 70/30% interface fraction were concentrated and tested for RT activity. Five microliters containing 0.5 to 6 μg of total protein as determined by Bradford assay (5) was added to make a 30-μl final reaction volume containing 25 mM Tris-Cl (pH ranging from 7.0 to 8.8), 20 mM dithiothreitol, 0.1 μM cold dGTP, 100 μg of poly(C) per ml, 10 μg of oligo$(dG)_{12-18}$ per ml, 3 U of RNasin (Promega Corp.), and 0.05 μCi of [α-^{32}P]dGTP (3,000 Ci/mmol; Amersham Corp.). Reaction mixtures were supplemented with $MgCl_2$, $MgSO_4$, and/or $MnCl_2$ as the sources of divalent cations at concentrations ranging from 0.1 to 20 mM. Assays were incubated for 0 to 2 h at temperatures ranging from 15 to 42°C. A range of 10 to 100 mM NaCl or KCl was used to determined the effect of these salts on RT activity. Incorporation was monitored by

spotting 15-μl aliquots onto DE81 filter paper (Whatman, Inc.), washing four times for 5 min each with 5% dibasic phosphate, washing in 95% ethanol for 1 min, and counting in an LS-230 scintillation counter (Beckman).

Peptide design and synthesis. The production of antibodies to Ty3-specific peptides facilitated characterization of Ty3-encoded proteins. Peptides were designed from protein domains predicted from three regions of the Ty3 genome. They were chosen to represent the amino- and carboxyl-terminal regions of the predicted *GAG3* protein and the RT domain of the predicted *POL3* protein. The peptides designed from these domains were designated GAG1, GAG2, and RT, respectively. The peptides were chosen for their hydrophilicity, as predicted by profiles of the amino acid sequence (26; SnAP program, by A. Goldin and G. Gutman, University of California, Irvine). The three peptides were synthesized by Multiple Peptide Systems and are as follows: (i) GAG1, NH_3-LTFRGRNDSHKLKNFC-COOH, Ty3-1 *GAG3* residues 30 to 44 (numbered from the initiator methionine); (ii) GAG2, NH_3-VRTRRSYNKPMSNHRNRRC COOH, Ty3-1 *GAG3* residues 235 to 252; and (iii) RT, NH_3-DKSQWTEKQDKAIDKC-COOH, Ty3-1 *POL3* residues 577 to 591 (this peptide was designed based on the Ty3-2 sequence; the Ty3-1 sequence has glutamate [E] instead of aspartate [D] at residue 590). The carboxyl cysteine residue of each of these peptides is not encoded by Ty3, but was used to couple the peptides to ovalbumin with the bifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (30, 33). The three peptide-ovalbumin conjugates were sent to Berkeley Antibody for production of antipeptide antibodies in rabbits. Reaction of each antiserum to its respective antigenic peptide was monitored by an enzyme-linked immunosorbent assay (15), using horseradish peroxidase-linked, goat anti (α)-rabbit immunoglobulin G (IgG) antibodies (Sigma Chemical Co.). Each respective preimmune serum was used as a negative control.

Affinity purification of antibodies. The antipeptide antibodies were affinity purified for use in the immunoblot and immunofluorescence analyses. The IgG fraction was isolated by passing each antiserum over a protein A column (Immunopure immobilized protein A gel; Pierce Chemical Co.) (20), followed by elution of the bound IgG with 0.1 M glycine (pH 2.8). The peak fractions were identified spectrophotometrically by determining A_{280}, adjusted to neutral pH, and dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4). The IgG fraction of each antiserum was then passed over separate Sulfolink coupling gel (Pierce) columns, to which the respective peptide had been conjugated through the cysteine residue (19). The antipeptide antibodies were eluted with 0.1 M glycine (pH 2.8). Peak fractions were identified by A_{280}, pooled, and dialyzed against PBS. Bovine serum albumin (BSA) was added to a concentration of 1% (wt/vol) to stabilize the IgG, sodium azide was added to a concentration of 0.02% to preserve sterility, and aliquots were stored at −20°C.

Immunoblot analysis. Concentrated VLP proteins from extracts of transformed yVB110 and the equivalent amount of interface protein from untransformed yVB110 control cells (approximately 20 μg of total protein) were fractionated on Laemmli SDS-polyacrylamide gels (12% acrylamide for the α-GAG1 analysis, 15% acrylamide for the α-GAG2 analysis, and 10% acrylamide for the α-RT analysis) and transferred to NitroScreen West membranes (DuPont, New England Nuclear Corp.) by the wet-transfer method (45). For the α-GAG2 immunoblot, the membrane-bound protein was also stained with a solution containing 0.5% Ponceau S (Sigma) and 1% acetic acid in order to visualize low-molecular-weight protein standards (Bethesda Research Laboratories) and then destained with water. The membrane-bound proteins were subjected to immunoblot analysis as previously described (22). Membranes were incubated with the appropriate affinity-purified antibody, washed, and incubated with ^{125}I-labeled protein A (ICN Pharmaceuticals Inc.). Proteins were visualized by exposure to Kodak XAR-5 film.

Immunofluorescence microscopy. Indirect immunofluorescence microscopy was performed as described by Pringle et al. (39). Cells of the Ty3-null strain, yVB110, or yVB110 transformed with pEGTy3-1 were grown in galactose-containing medium at 30°C to an OD_{600} of 0.2 to 0.3. Nine milliliters of each sample was added to 1 ml of 37% formaldehyde, fixed for 90 min at 30°C, washed with 0.1 M potassium phosphate (pH 6.5), resuspended in sorbitol-containing buffer (0.1 M Tris [pH 8], 5 mM EDTA, 25 mM dithiothreitol, 1 M sorbitol), and incubated for 5 min at 30°C. The cells were then collected by centrifugation and digested with Zymolyase (0.1 mg/ml; 100,000 lytic units/g [100T]; ICN) and glucuronidase (270 U/ml) in 0.5 ml of buffer (0.1 M potassium phosphate [pH 5.8], 1.2 M sorbitol), for 75 to 90 min at 30°C. The cells were then washed and suspended in 200 to 300 μl of 0.1 M potassium phosphate (pH 6.5). Thirty microliters of each sample was applied to polylysine-treated microscope slides and fixed in methanol and acetone at −20°C for 6 min and 30 s, respectively. The fixed cells were blocked for 5 min with 0.1% BSA in PBS, incubated with affinity-purified α-GAG1 (diluted 1:100 with 0.1% BSA in PBS) at room temperature for 1 to 2 h, washed with 0.1% BSA in PBS, and incubated with fluorescein 5-isothiocyanate (FITC)-conjugated goat α-rabbit IgG antibodies (Sigma) in the dark at room temperature for 1 to 2 h. The cells were then washed with PBS, and the nuclear DNA was stained with a 0.1% 4′,6-diamidino-2-phenylindole (DAPI) solution for 5 min and washed again with PBS. The slides were mounted and sealed in medium containing *p*-phenylenediamine and examined by epifluorescence with a Zeiss fluorescence photomicroscope. Pictures were taken with Kodak T-Max (ASA 400) film.

Electron microscopy. Cells from yeast strain yVB110 transformed with pEGTy3-1 were grown in uracil-minus medium containing 2% galactose to an OD_{600} of 0.5, at which point an equal volume of YPG (1% yeast extract, 1% Bacto Peptone, 2% galactose) medium was added. The culture was allowed to double to an OD_{600} of 0.5, and then cells were fixed in 4% glutaraldehyde for at least 24 h. The cell walls were digested with Zymolyase (2 mg/ml; 100T) in 0.1 M phosphate citrate buffer (pH 5.8) at 37°C for 45 min and washed in 0.1 M potassium acetate (pH 6.1). The cells were then incubated in 2% osmium tetroxide for 1 h. Finally, the cells were washed, dehydrated with increasing concentrations of ethanol, and embedded in Spurr resin (43). Sections were stained in uranyl acetate and lead citrate and viewed in a Philips EM 300 electron microscope.

RESULTS

Isolation of VLPs. To produce regulated, high levels of transcripts from a transpositionally active Ty3 element, yeast cells were transformed with a high-copy-number plasmid that contained the *GAL1-10* upstream activating sequence (28) fused to the promoter of Ty3-1 (21). Extracts of cells grown in galactose-containing medium were fraction-

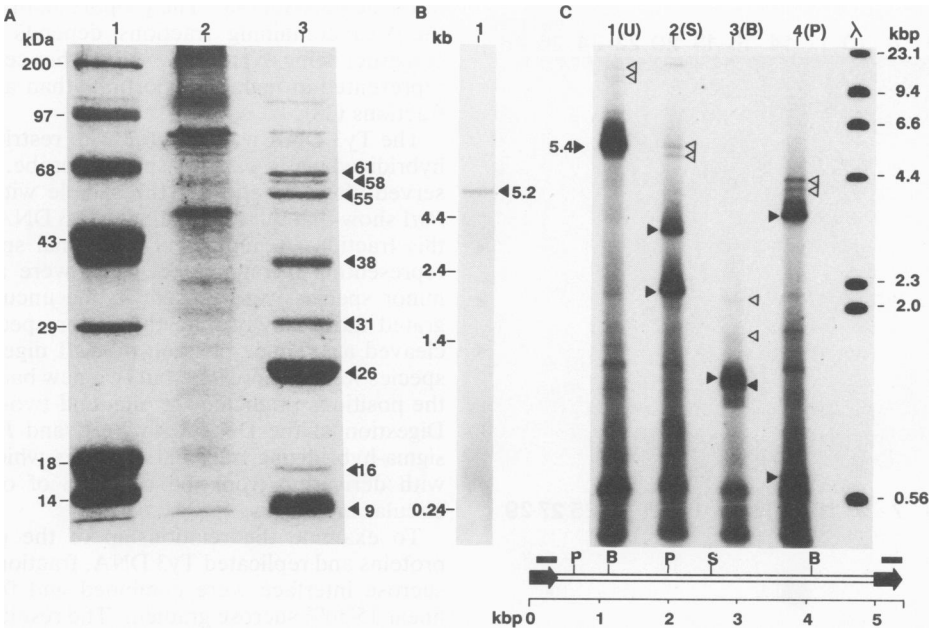

FIG. 1. Analysis of Ty3 proteins and nucleic acids copurifying on sucrose step gradients. Extracts were isolated from AGY9 cells (for protein analysis) or yVB110 cells (for nucleic acid analyses) grown on galactose-containing medium and fractionated on sucrose step gradients. (A) Ty3 structural proteins. Fractions from the 70/30% interface were concentrated by ultracentrifugation, subjected to electrophoresis on an SDS–10% polyacrylamide gel, and stained with Coomassie blue. The molecular weight size markers (Bethesda Research Laboratories) are indicated in lane 1. Proteins were isolated from nontransformed cells (lane 2) and cells which were transformed with pEGTy3-1 (lane 3). Approximately 5 μg of total protein was loaded per lane. Ty3-specific proteins and apparent molecular masses are indicated by arrowheads. (B and C) Isolation of Ty3 RNA and DNA. Nucleic acids were extracted from the 70/30% interface and analyzed for the presence of Ty3-specific RNA and DNA. Panel B shows Northern blot analysis of a sample from Ty3-1 transformants after treatment with DNase I. RNA was detected by hybridization to a radiolabeled probe specific to the Ty3 internal domain (to which the Ty3-null strain yVB110 RNA shows no hybridization). Panel C shows the pattern produced by restriction enzyme digestion of Ty3 DNA present at the sucrose step gradient 70/30% interface. VLP DNA, undigested (U) (lane 1) or digested with *Sal*I (S) (lane 2), *Bgl*II (B) (lane 3), or *Pst*I (P) (lane 4), was hybridized to a sigma-specific probe. Size markers (λ) were lambda DNA digested with *Hin*dIII and end labeled with [γ-^{32}P]ATP. The restriction map of Ty3 for the enzymes used in this analysis is shown at the bottom. Sigma elements are represented as solid arrows and the sigma sequences which were used as a probe are indicated by the solid bar. Solid arrowheads denote the positions of restriction fragments predicted for a 5.4-kbp, linear Ty3 DNA species. Open arrowheads indicate sigma-hybridizing fragments predicted for one- and two-LTR, circular DNA species.

ated over sucrose step gradients, and fractions containing Ty3-specific proteins and nucleic acids were identified by gel electrophoresis followed by Western (immunoblot), Northern, and Southern blot analyses.

Proteins present in the sucrose step gradient fractions were concentrated by centrifugation and fractionated by SDS-polyacrylamide gel electrophoresis (Fig. 1A). The 70/30% interface fraction was enriched for Ty3-specific proteins. Comparison of protein patterns of fractionated extracts from Ty3-null cells (yVB110) with those of extracts from cells expressing high levels of Ty3-1 RNA allowed identification of Ty3-specific proteins with apparent molecular sizes of 38, 31, and 26 kDa in Coomassie-stained polyacrylamide gels (23). Other Ty3-specific proteins were obscured by a background of cellular proteins in the yVB110 extract. However, Ty3 proteins were the major constituents of the concentrated 70/30% interface fractions from pEGTy3-1-transformed AGY9 cells; thus, extracts from this strain allowed detection of other Ty3-specific proteins. Fractions from the 30% step and both interfaces contained about 1/10 of the total protein in the extract and all of the detectable *GAG3* protein according to Western blot analysis using the α-GAG1 antibody (described below). In addition to the 38-, 31-, and 26-kDa proteins, Ty3-specific proteins with apparent molecular sizes of 61, 58, 55, 16, and 9 kDa were identified by comparison of extracts from yeast strain AGY9 (Fig. 1A, lane 2) with extracts from these cells overexpressing Ty3-1 RNA (lane 3). Equivalent amounts of protein were loaded in each lane. By analogy with the retroviral core, the predominant protein constituent(s) of the Ty3 nucleoprotein complex were expected to be encoded by *GAG3*. The relative abundance and apparent molecular weights of the 26- and 9-kDa proteins suggested that these proteins are major products of processing of the full-length *GAG3* polyprotein. Proteins of the sizes predicted from the *POL3* reading frame, including the 61- and 58-kDa proteins shown previously to be encoded by the putative IN domain (22), were produced at a lower level than the *GAG3*-encoded proteins, as anticipated by their derivation from a *GAG3-POL3* fusion (16).

Nucleic acids were extracted from fractions representing the 70/30% interface, treated with DNase I or RNase A, and analyzed on Northern (Fig. 1B) and Southern (Fig. 1C) blots, respectively. These fractions contained a 5.2-kb RNA and a 5.4-kbp DNA that hybridized to Ty3- and sigma-specific radiolabeled probes, respectively. Overall, approximately 1% of the total OD_{260} recovered from the gradient was in the 30% sucrose step and interface fractions. Analysis with Ty3- and actin-specific probes showed that these fractions contained about one-third of the 5.2-kb Ty3 RNA and about 1/20

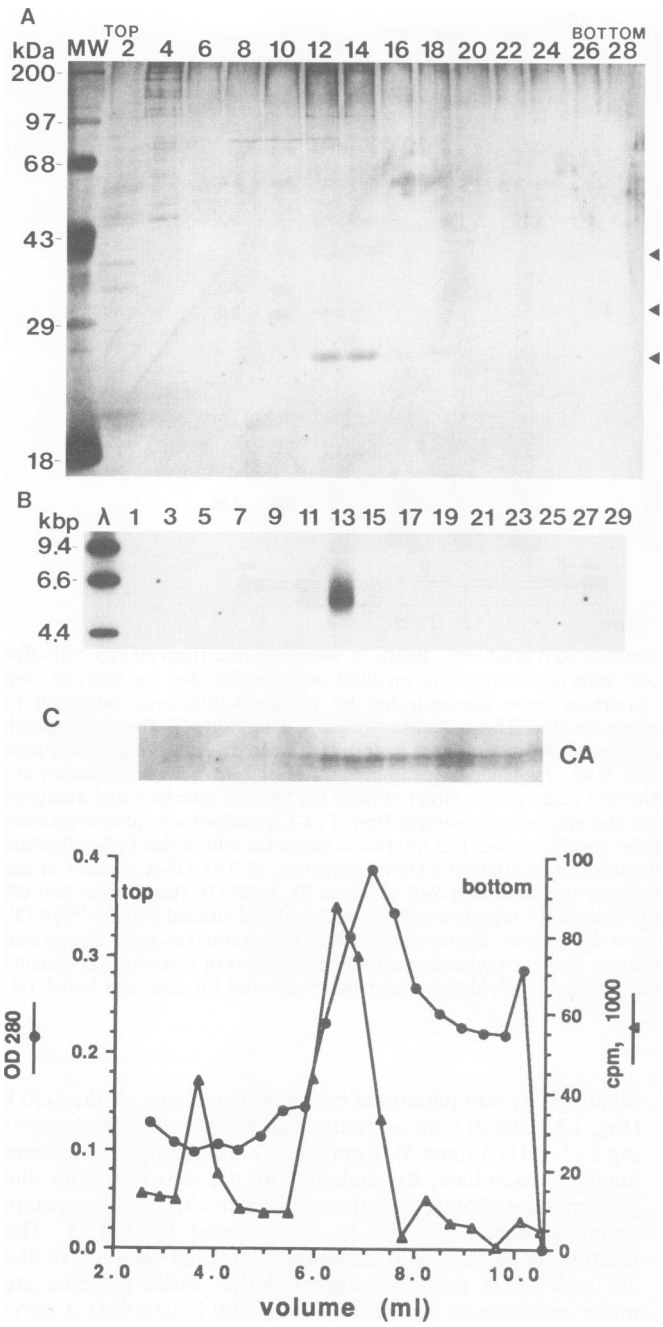

FIG. 2. Analysis of linear sucrose gradients for Ty3 proteins and DNA. Samples isolated on the sucrose step gradient were resedimented over a linear 15/50% sucrose gradient (A and B). (A) Ty3 protein analysis. Even fractions, 2 through 28, were analyzed on SDS–10% polyacrylamide gels and visualized by silver staining. Arrowheads indicate proteins corresponding to the 26-, 31-, and 38-kDa species identified on step gradients. (B) Southern analysis. Nucleic acids were extracted from odd fractions as described in Materials and Methods and analyzed on a Southern blot. The major DNA species shown here corresponded to a linear, 5.4-kbp species. Nucleic acids were visualized as described previously by hybridization to a radiolabeled probe specific to the internal domain of Ty3. (C) Determination of Ty3 particle size. Ty3 VLPs from the 70/30% step gradient interface were resedimented, in parallel with poliovirus, on a 10/30% linear sucrose gradient. The peak of total protein, representing primarily VLPs, is shown as a plot of OD_{280}; the peaks of poliovirus proteins were identified by determining the radioactivity of aliquots of gradient fractions in a scintillation counter. Both peaks were confirmed by characteristic patterns of proteins on SDS-polyacrylamide gels (38), and the position of sedimentation of the 26-kDa Ty3 protein as determined by silver staining is shown.

of the actin RNA (38). The proportion of Ty3 RNA found in the VLP-containing fractions depends on the particular construct being expressed. Some other cellular messages are represented at higher proportions than actin RNA in these fractions (38).

The Ty3 DNA was digested with restriction enzymes and hybridized with a sigma-specific probe. The patterns observed after digestion of the sample with *Sal*I, *Bgl*II, and *Pst*I show that full-length, linear Ty3 DNA is a major form in this fraction. A number of additional species, presumably representing incomplete cDNAs, were also present. Two minor species were present in the uncut DNA which migrated more slowly than the major species. In Ty3 DNA cleaved at a single position by *Sal*I digestion, these minor species were not present, but two new bands were present at the positions predicted for one- and two-LTR linear forms. Digestion of the DNA with *Bgl*II and *Pst*I also produced sigma-hybridizing fragments of sizes which were consistent with derivation from the cleavage of one- and two-LTR circular molecules.

To examine the relationship of the putative structural proteins and replicated Ty3 DNA, fractions from the 70/30% sucrose interface were combined and fractionated over a linear 15/50% sucrose gradient. The results of the analysis of these gradient fractions for Ty3 proteins and DNA are shown in Fig. 2A and B, respectively. The 5.4-kbp Ty3 DNA and the 5.2-kb Ty3 RNA (not shown) were found in the same linear gradient fractions that contained the peak of Ty3-specific proteins. Thus, in cells expressing high levels of Ty3 transcripts, Ty3-specific RNA, DNA, and proteins cofractionate on sucrose gradients. These results are consistent with the existence of a Ty3 nucleoprotein complex, within which replication takes place.

Velocity sedimentation analysis of the Ty3 VLPs compared with poliovirus standards showed that the VLPs are approximately 156(±5)S in size (Fig. 2C). This value is about the same as the 160S measured for Moloney murine leukemia (MoMLV) virus core (4). The particle size for Ty1 has been reported at 175S (35) and 275S (18). In our experiments, although a peak consistent with 156S was observed, Ty3 proteins also occurred further down in the gradient. A similar heterogeneity in the sedimentation of Ty1 might explain the apparent discrepancy in sizes reported.

Characterization of Ty3 RT activity. Fractions from the sucrose step gradient concentrated from the 70/30% interface containing Ty3 nucleic acids and proteins were examined for the presence of RT activity by using an exogenous oligo(dG)-poly(C) primer-template assay. Early attempts using an oligo(dT)-poly(A) primer-template assay were frustrated by a high level of nonspecific incorporation. Because one potential source of background activity was endogenous Ty1 elements, RT activity was also assayed in strain AGY9, which contains a disruption of the *SPT3* gene. The product of this gene is essential for efficient Ty1 expression. Extracts were prepared from AGY9 cells and from AGY9 cells transformed with pEGTy3-1 that had been cultured in medium containing galactose and fractionated on step gradients. Interface fractions in which Ty3 proteins and nucleic acids were observed also contained RT activity (Fig. 3A).

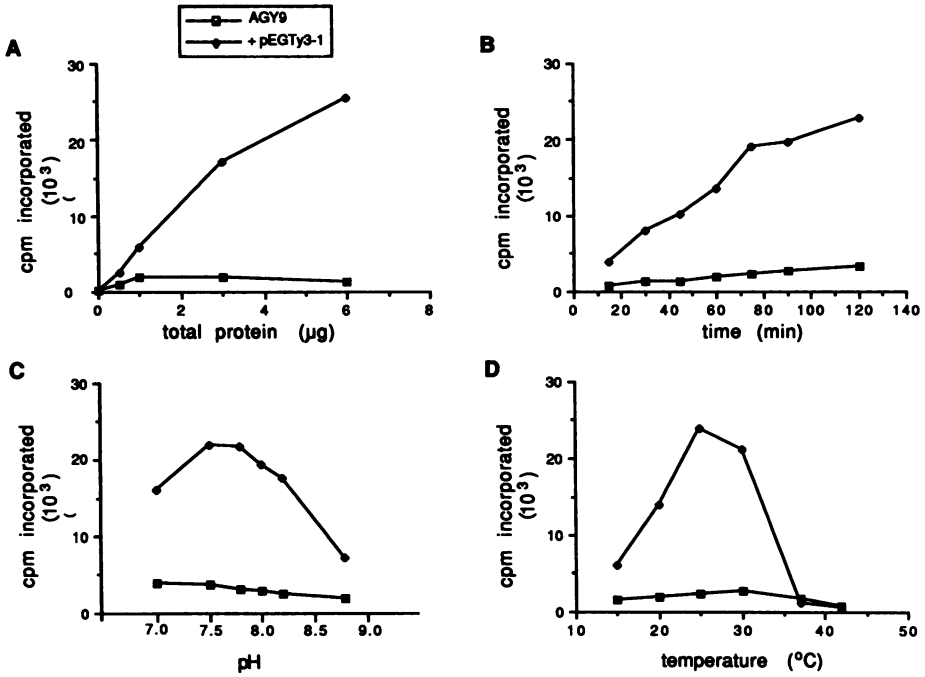

FIG. 3. Characterization of Ty3 RT. Extracts from *spt3* strain AGY9 or strain AGY9 transformed with pEGTy3-1, fractionated on sucrose step gradients and concentrated by ultracentrifugation, were analyzed for RT activity by using oligo(dG)-poly(C) as an exogenous assay primer and template. The data presented are from a representative experiment. RT activity is shown as a function of extract protein concentration (A), time (B), pH (C), and temperature (D). Assays were performed at 30°C and pH 7.8 unless otherwise noted. In assays represented in panels B through D, reaction mixtures contained either 1 or 3 μg of total protein from AGY9 cell extracts or these cells expressing Ty3-1, respectively, in order to normalize assays per cell number, taking into account increased protein levels present in cells overexpressing Ty3. As indicated in panel A, however, activity in AGY9 alone was uniformly low at the protein concentrations tested even when normalized to protein concentration.

This activity was dependent on magnesium and was highest at a Mg^{2+} concentration of 15 mM. $MgSO_4$ gave approximately 40% higher activity than did comparable levels of $MgCl_2$. Manganese did not substitute as a divalent cation. Incorporation of radiolabeled dGTP was dependent on addition of both exogenous primer and template unless the reaction was supplemented with 100 mM each dATP, dCTP, and dTTP, thus allowing incorporation into an endogenous substrate (8). Reverse transcription of the exogenous template at 30°C continued steadily for at least 2 h (Fig. 3B). The optimal pH for the Ty3-specific activity was between 7.5 and 7.8 (Fig. 3C). Ty3 RT activity increased with increasing temperature up to 25°C and decreased slightly between 25 and 30°C (Fig. 3D). Between 32 and 37°C, activity decreased rapidly in a linear fashion (8). Addition of NaCl, KCl, or potassium glutamate did not significantly stimulate activity, and concentrations above 25 mM salt resulted in decreased activity.

Immunoblot analysis of Ty3 VLPs. Ty3-encoded proteins were further characterized by immunoblot analysis of VLP preparations, using antipeptide antibodies directed against two regions of the *GAG3* protein (α-GAG1 and α-GAG2) and a region of the *POL3* protein predicted to be present in the RT (α-RT). To verify that the 38-, 31-, 26-, and 9-kDa proteins identified in the SDS-polyacrylamide gel analysis were encoded within *GAG3*, antibodies were generated against peptides represented near the amino-terminal and the carboxyl-terminal regions of the predicted *GAG3* protein. The *GAG3* amino-terminal peptide, GAG1, consisted of *GAG3*-encoded residues 30 to 44. The *GAG3* carboxyl-terminal peptide, GAG2 (residues 235 to 252), was derived from the region immediately preceding the single copy of the $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ motif found in the carboxyl-terminal region of retroviral NC proteins. The results of the immunoblot analyses using the α-GAG1 and α-GAG2 antibodies are shown in Fig. 4A and B. The α-GAG1 antibodies reacted with the 38-, 31-, and 26-kDa proteins and a minor Ty3 protein with apparent molecular mass of 39 kDa. The α-GAG2 antibodies reacted with the 38-kDa protein and also with a 9-kDa protein. This antibody did not react clearly with minor components. The broadness of the band representing the 9-kDa protein may be due the general failure of small proteins to resolve well under these electrophoretic conditions or could reflect heterogeneity in processing.

The RT peptide (*POL3-1* residues 577 to 591) was derived from a region of *POL3-2* which encodes a protein that is homologous to retroviral RT proteins. The α-RT antiserum reacted specifically with a protein of 55 kDa in the immunoblot analysis of the VLP fraction (Fig. 4C). Previous IN studies suggested the presence of a minor 115-kDa RT/IN protein (22). A protein of this size was not detected by the α-RT antibody used in the current study. However, the pattern generated by the α-RT antibody was relatively weak, and interaction with a minor protein might not have been observed. Figure 5 shows a schematic diagram of the proposed derivation of proteins identified by Western blot analysis.

Immunofluorescence of cells overexpressing Ty3. Ty3 nucleoprotein complexes were examined by immunofluorescence (Fig. 6). Strains yVB110 and yVB110 transformed

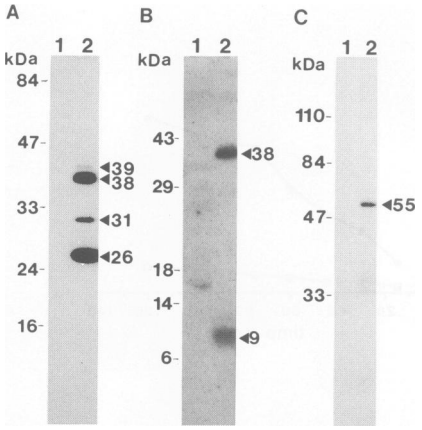

FIG. 4. Immunoblot analyses of Ty3 VLPs. Extracts were prepared from the Ty3-null strain (yVB110; lanes 1) and from galactose-induced yeast cells containing the pEGTy3-1 plasmid (lanes 2) and were fractionated over sucrose step gradients to concentrate VLPs as described in Materials and Methods. Proteins were fractionated on SDS-polyacrylamide gels, transferred to Nitroscreen West membranes, and reacted with affinity-purified antibodies. The antibody-protein complexes were visualized by incubation with ^{125}I-protein A and exposure to X-ray film. The antibodies used were α-GAG1 (SDS–12% polyacrylamide gel) (A), α-GAG2 (SDS–15% polyacrylamide gel) (B), and α-RT (SDS–10% polyacrylamide gel) (C). The molecular masses of protein standards are indicated at the left of each autoradiogram. The deduced masses of the predominant reactive proteins are shown in kilodaltons at the right.

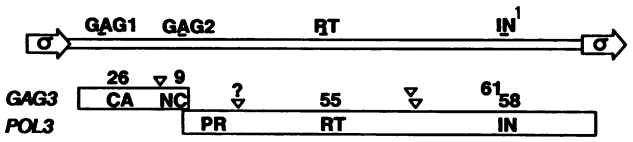

FIG. 5. Coding domains of Ty3-1. The Ty3 element is diagrammed at the top. The 340-bp sigma elements (open arrows) flank the 4.7-kbp internal domain (narrow open box). The wide open boxes below the Ty3 element represent Ty3 ORFs. *GAG3* is 290 codons in length starting at the initiator methionine. *POL3*, the second ORF (Ty3-1), is 1,270 codons in length and is in the +1 frame relative to *GAG3*. The predicted protein sequences have been compared with conserved domains in retrovirus proteins and regions of homology are indicated: CA, capsid; NC, nucleocapsid; PR, aspartyl protease; RT, reverse transcriptase; and IN, integrase. The positions of GAG1, GAG2, RT, and IN peptides used to generate antibodies are indicated above the internal domain ([1]described by Hansen and Sandmeyer [22]). The apparent molecular weights (in thousands), as determined by Western blot analysis, of proteins encoded by each domain are listed above the ORFs. The open arrowheads indicate putative proteolytic processing sites inferred from the sizes of the Ty3 proteins and positions of the protein-coding regions in Ty3.

with pEGTy3-1 were grown in galactose-containing medium. Cells were examined by differential interference contrast (DIC) (Fig. 6A and D) and by immunofluorescence microscopy, using the α-GAG1 antiserum, which reacts with the 38-, 31-, and 26-kDa proteins. Protein-antibody complexes were visualized by reaction with FITC-conjugated goat α-rabbit IgG antibodies (Fig. 6B and E). Nuclear DNA was stained with DAPI (Fig. 6C and F).

Strain yVB110 alone shows only background fluorescence (Fig. 6B). In contrast, brightly fluorescent Ty3-specific protein aggregates can be seen in approximately 1 in 10 of the cells expressing Ty3-1 (Fig. 6E). The positions of the Ty3 aggregates can be superimposed on cellular protrusions apparent in the DIC photographs (Fig. 6D and E). These protrusions were even more striking in micrographs of cells which also contained a galactose-inducible *GAL4* gene but were not apparent in induced cells containing a single integrated copy of the galactose-inducible Ty3 element (23).

The patterns of fluorescent complexes and nuclear staining were examined to determine whether the Ty3 nucleoprotein complexes were present in any fixed relationship to the nucleus (Fig. 6E and F). Although occasional colocalization of immunofluorescence and DAPI-staining material was observed, the vast majority of these clusters appeared to be cytoplasmic. The sensitivity of this method did not allow visualization of individual particles.

Electron microscopy. Nontransformed and pEGTy3-1-transformed yVB110 cells were grown in medium containing galactose and examined by electron microscopy for the presence of VLPs. This experiment showed that overexpression of Ty3 correlated with the appearance of large clusters of particles approximately 50 nm in diameter (Fig. 7). These particles resemble those previously described for Ty1. The percentage of cells observed to have clusters was about one-third to one-fifth the percentage of cells observed to have immunoreactive material. Although scattered particles were observed in some fields, no clusters were observed in the Ty3-null cells (38). The majority of the particles were clustered in the cytoplasm and were heterogeneous in appearance.

DISCUSSION

In this work, analysis of fractionated cell extracts showed that cells transcribing the yeast retrotransposon Ty3 contained a 5.2-kb RNA, a 5.4-kbp, linear DNA, and proteins encoded by Ty3. Further, these macromolecules and RT activity cofractionated in sucrose gradients at a position consistent with a macromolecular complex, separated from the positions of control mRNA for actin and many cytoplasmic proteins. The most simple interpretation of these data is that a Ty3 macromolecular complex, which corresponds to the VLPs visualized by electron microscopy, exists in cells expressing high levels of Ty3 RNA. In addition, immunoprecipitations using α-GAG1 antibodies coprecipitated Ty3 RNA and DNA from total cell extracts and from sucrose gradient fractions (38). The macromolecular complex containing Ty3 proteins and nucleic acids is referred to as the Ty3 VLP or nucleoprotein complex and is predicted to be functionally similar to the retroviral core.

The *GAG3* ORF is translated into a protein of apparent mass of 38 or 39 kDa. The major protein constituents of the wild-type Ty3 VLP are a 26-kDa and a 9-kDa protein, encoded by *GAG3*. These proteins reacted with antibodies directed at antigenic determinants located at the amino-terminal and carboxyl-terminal regions of the *GAG3* protein, respectively. We conclude that the major 38-kDa or minor 39-kDa protein which reacted with antibodies directed at both the amino and carboxyl regions is the full-length *GAG3* protein. The 39-kDa protein could be the complete *GAG3* translation product, a modified form of the 38-kDa protein, or a protein derived from maturation of the *GAG3-POL3* fusion protein. The *GAG3* ORF predicts a protein with a molecular mass of about 34 kDa; nevertheless, analysis of other small, basic proteins, such as retroviral *gag*-encoded proteins, has shown that they can migrate anomalously

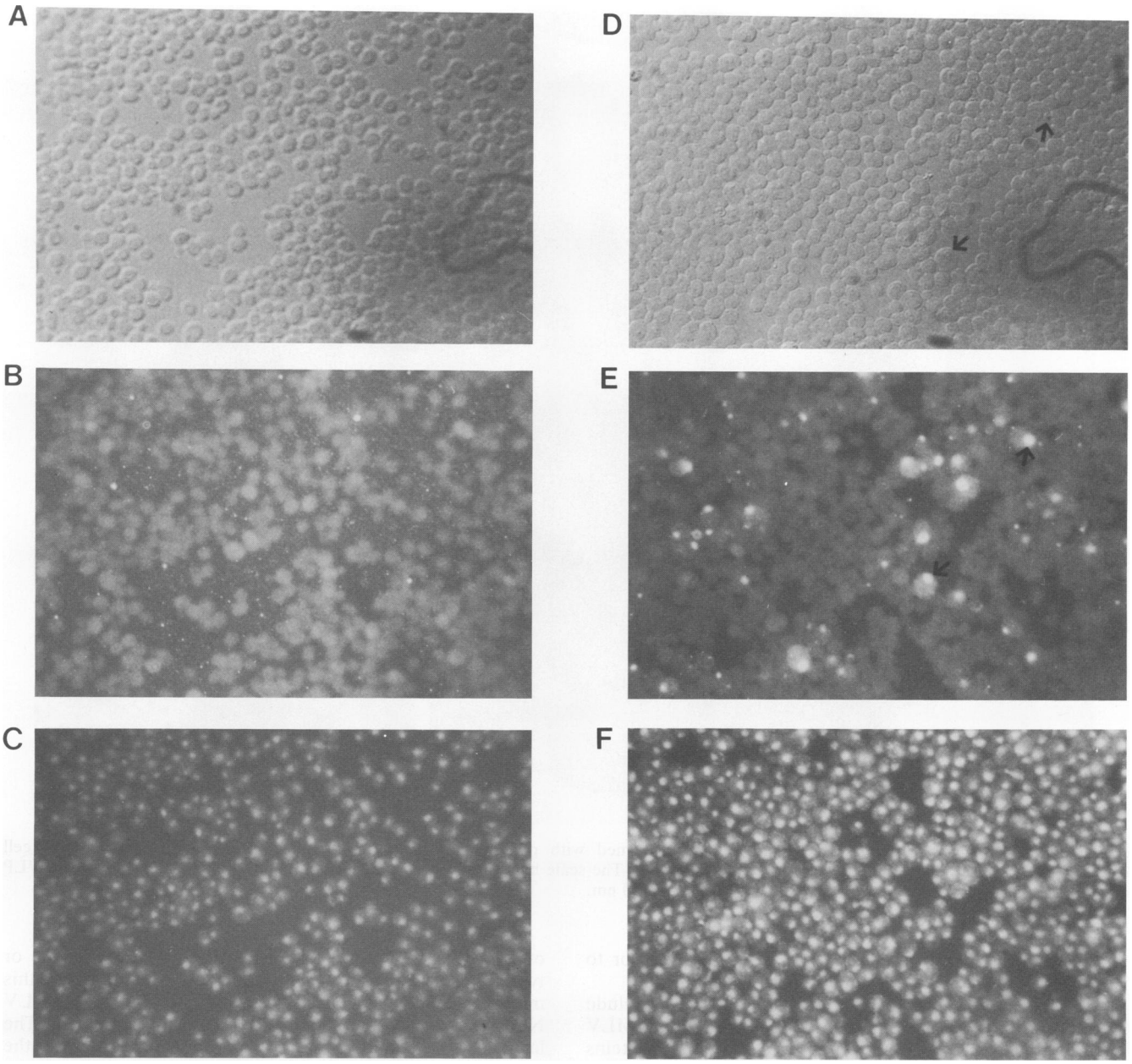

FIG. 6. Immunofluorescence microscopy. Yeast cells (yVB110 [A to C] and yVB110 transformed with pEGTy3-1 [D to F]) were grown in galactose-containing medium and prepared for immunofluorescence as described in Materials and Methods. The cells were fixed, and intact cells were visualized microscopically by DIC (A and D), α-GAG1 rabbit antibodies and secondary α-rabbit IgG antibodies conjugated to FITC (B and D), and DAPI (C and F). Arrows denote representative cells in which cellular protrusions and Ty3 aggregates can be superimposed.

slowly, even under denaturing conditions similar to those used in these experiments (11, 25). The discrepancy between the size of the predicted *GAG3* protein and the apparent mass of 38 kDa would be consistent with these findings. The band representing a protein of apparent mass of 31 kDa could be attributable to minor unprocessed or processed forms, degradation, or modification.

The levels of *GAG3* proteins relative to the levels of the RT and IN proteins can be approximated from analysis of VLP proteins isolated from strain AGY9 overproducing Ty3 proteins. This analysis (taking into account the mass of proteins and assuming comparable staining with Coomassie blue of the different proteins) gives a *GAG3/POL3* ratio of 13:1 (8). The relatively high ratio of *GAG3*-encoded protein to *POL3*-encoded protein suggests a structural role. The majority of *GAG3*-encoded protein is produced by translation and termination at the end of the *GAG3* ORF; the reduced amounts of *POL3* proteins are produced as a *GAG3-POL3* fusion protein. Although a *GAG3-POL3* fusion protein has not been detected, the Ty3 VLPs that we studied were produced over long periods of galactose induction and are a heterogeneous population representing various stages of maturation. The ratios of various Ty3 proteins in the particle are likely to depend on the genetic background of the

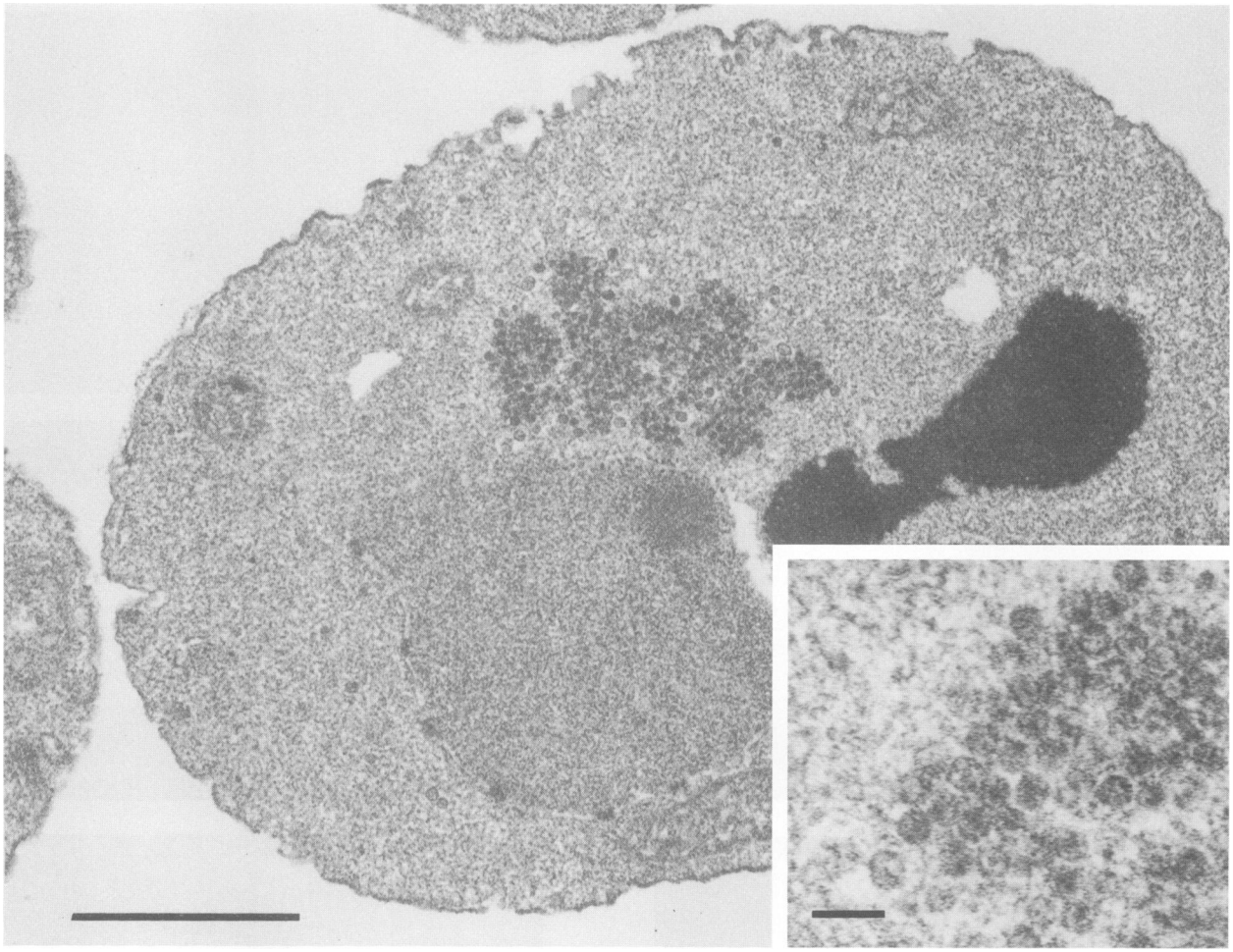

FIG. 7. Electron micrographs of yVB110 cells transformed with pEGTy3-1 and grown in galactose-containing medium. A cell overexpressing Ty3 RNA is shown at ×32,000 magnification. The scale bar represents 1 μm. The inset shows an enlargement of the VLP cluster at ×95,000 magnification. The scale bar represents 100 nm.

strain and probably also on the time of induction prior to isolation.

The common *gag*-encoded proteins of retroviruses include MA (e.g., murine leukemia virus [MLV] p15), CA (MLV p30), and NC (MLV p10) proteins, with additional proteins encoded (e.g., MoMLV p12 and Rous sarcoma virus PR), depending on the particular virus (reviewed in references 10, 12, 32, and 46). These proteins are produced at levels about 20 times the level of the catalytic proteins. Because of similarity in size, abundance, aspects of composition, presence of a partially conserved CA motif (48), and likely functional similarity to the retroviral CA protein, we have designated the 26-kDa protein the Ty3 CA. Although we could not detect a membrane association of Ty3 VLPs, our analysis does not exclude the possibility that the 26-kDa protein performs some particle formation or membrane association functions performed by retroviral MA.

The Ty3 VLP protein of an apparent molecular mass of 9 kDa is similar in size to the NC protein of retroviruses. These proteins migrate with apparent molecular masses of 10 to 15 kDa but are actually somewhat lower in mass, as determined from DNA sequence analysis. The MoMLV NC protein is composed of 60 residues and is encoded at the downstream end of the *gag* gene. The MoMLV NC includes one copy of the $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ motif found in one or two copies in all retroviral NC proteins. In MoMLV, this motif is encoded 21 codons from the end of *gag*. MoMLV NC includes 14 basic residues and six acidic residues. The last 60 *GAG3* codons contain this motif 10 codons from the downstream end and encode 17 basic residues and 4 acidic residues. (The region just upstream encodes a disproportionate concentration of acidic residues not observed in MoMLV *gag*, so the entire protein may not have a basic charge as in the case of MoMLV NC.) The 9-kDa protein was designated the Ty3 NC protein.

Analysis of the Ty3 VLPs revealed that proteins encoded by the *POL3* gene are represented in several species. The RT domain occurred in a 55-kDa protein which is derived from the region amino terminal to IN. The size of this protein would coincide with the approximate size of a protein encoded from the region just downstream of a 78-bp sequence that is present as a tandem repeat in Ty3-2 (21) up to the beginning of the region predicted to encode the amino terminus of Ty3 IN. A 115-kDa species that reacts with the α-IN antibody (22) could represent an RT/IN fusion analogous to the RT heterodimer observed for avian leukosis and sarcoma viruses. Whether one or both of these species is required for replication and integration is not known, but the

ability of Ty3-2 (which encodes a truncated IN) to replicate is much reduced (8, 38). Ty3 protein containing the IN domain migrates as a doublet (61 and 58 kDa) in immunoblot analysis of Ty3 VLPs. The smaller protein is more difficult to detect in immunoblot analysis of whole cell extracts (29). This doublet is likely to result from differential processing at the amino terminus of the IN protein (22). A protein of apparent molecular mass of 16 kDa was observed in extracts of cells expressing Ty3-1. The size and relative abundance of this species, as well as preliminary immunoblot analysis (8), are consistent with this protein being encoded by the *POL3* PR domain.

Proteins encoded in *GAG3* have similarity to those encoded in the *gag* gene of retroviruses, and proteins in *POL3* are related to those encoded by retroviruses and gypsylike elements. Because the gypsylike elements are redundant and, in many cases, degenerate, it has been difficult to identify active members of these families for analysis. This work presents identification of the major components of the nucleoprotein complex of a transpositionally active member of this family for the first time. Despite the fact that Ty3 has unusual position specificity, the protein and nucleic acid composition of the particle presented here support the model that these intracellular elements assemble VLPs and transpose by processes related to those used by retroviruses.

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