BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 166–171 (1998)
ARTICLE NO. RC988402

Secretion of Heterologous Proteins from *Schizosaccharomyces pombe* Using the Homologous Leader Sequence of *pho1*+ Acid Phosphatase

Joris Braspenning,[1] Wolfgang Meschede, Antonio Marchini, Martin Müller,[2] Lutz Gissmann, and Massimo Tommasino

Deutsches Krebsforschungszentrum, Angewandte Tumorvirologie, INF 242, 69120 Heidelberg, Germany

Received March 3, 1998

In this study we report the use of the *S. pombe* leader sequence of *pho1*+ acid phosphatase (Elliott *et al., J. Biol. Chem.* 216, 2916–2941, 1986) for the secretion of heterologous proteins into the medium. The green fluorescent protein (GFP) and the Human Papillomavirus (HPV) type 16 E7 protein are normally not secreted; fusion of the *S. pombe pho1* leader peptide (SPL) to GFP and HPV 16 E7 resulted in an efficient secretion of these proteins although the latter contains a nuclear targeting sequence. These data suggest that SPL fused constructs could be applied for the production of other recombinant proteins using the *S. pombe* expression system. Furthermore, since GFP retains its intrinsic fluorescence during the secretion, this system may be useful to study the secretory pathway of fission yeast *in vivo.*

Key Words: **secretory pathway; *S. pombe;* GFP; HPV; 16 E7; intracellular targeting.**

Yeast cells are well suited for the production of heterologous eukaryotic proteins. They facilitate post-translational processing of polypeptides, such as folding, phosphorylation and glycosylation. *Saccharomyces cerevisiae* is commonly used since as a food organism it is highly acceptable for the production of pharmaceutical proteins. However, other yeasts such as *Pichia pastoris, Kluyveromyces lactis* and *Schizosaccharomyces pombe* are also used for the production of recombinant proteins. The latter is less studied as an expression system, presumably due to a lack of tightly regulatable promoters. Only after the development of inducible expression vectors (1), *Schizosaccharomyces pombe* was used for the expression of foreign proteins (2) (3) (4) (5). Since overexpressed recombinant proteins can lead to insoluble and inactive intracellular products, we were interested in developing a secretion system for heterologous proteins in *S. pombe.* A signal peptide was identified by Elliott et al. (6) which directs secretion of acid phosphatase in *S. pombe.* We tested if this *pho1* signal peptide is able to direct different recombinant proteins to a secretory pathway. To monitor the secretion of proteins in vivo we used a visual marker, the green fluorescent protein (GFP). This protein was isolated from the jellyfisch *Aequorea victoria* and yields a bright green fluorescence when illuminated with blue or UV light. Expression of this protein in other systems has demonstrated the ability of GFP to retain its fluorescence (7) (8)(9) (10). We have also tested if a secretory leader peptide can override the nuclear localization sequence of certain proteins. We have chosen to test the Human papillomavirus (HPV) type 16 E7 protein which is an acidic phosphoprotein of 98 amino acids and localized within the nucleus in higher eukaryotic cells and *S. pombe* (11) (12) (13) (14) (15) (16). The *pho1* leader sequence of *S. pombe* (SPL) was sufficient to translocate both recombinant GFP and the E7 protein for secretion into the growth medium. The present study also demonstrates the utility of GFP to follow proteins *in vivo* during their translocation within the yeast cell.

MATERIALS AND METHODS

Strains, media and growth conditions. The yeast Schizosaccharomyces pombe, strain leu1- 32 h-, was used for all experiments. Cells were cultured aerobically in erlenmeyer flasks at 30°C in selective synthetic medium (EMM2) supplemented with thiamine to inhibit expression of the heterologous proteins. Growth of cells was monitored photometrically using the following calculation: Cells/ml = $OD_{595} \times 5.5 \times 10^6$. Expression of proteins was induced after thiamine removal as described by Maundrell (1993). Medium reagents were from Sigma except thiamine which was from Merck.

[1] Corresponding author. Fax: +49-6221-424932; E-mail: j.braspenning@dkfz-heidelberg.de.

[2] Loyola University Medical Center, Department of Microbiology and Immunology, 2160 South First Avenue, Maywood, IL 60153, USA.

0006-291X/98 $25.00

Vector constructions. The unfused genes, GFP and HPV 16 E7 (17), were PCR amplified, cloned into Bluescript-SK and after sequencing sub-cloned into pREP (1) as shown in Figure 1. The thiamine-repressible expression vector pREP3 and the pho1 signal peptide of 18 amino acids (6) of S. pombe provided the basis for constructing the SPL (S. pombe leader) containing vector. The signal peptide pho1 encoding sequence (6) was chemically synthesized with additional 5′-BamHI site and made double stranded using Pol1K and specific primers. Two constructs were made either (A) 5′-BamHI and 3′-NsiI, SalI or (B) 5′-BamHI and 3′-NcoI, SalI. The multiple cloning site of pREP3 was modified and the construct is further referred to as L20. Fragment (A) was cloned together with the 16 E7 coding sequence (NsiI and SalI) into the BamHI and SalI digested S. pombe vector L20 to obtain the leader-E7 hybrid polypeptide. Fragment (B) was cloned into Bluescript-SK using the sites BamHI and SalI. This vector was NcoI and SalI digested for ligation with GFP which was isolated from the NcoI and SalI digested vector pEGFP-C1 (CLONTECH), to obtain GFP in frame with the leader encoding sequence. The leader fused fragment was sub-cloned in L20 using the sites BamHI and SalI for expression in yeast (Fig. 1).

Expression and detection of proteins. The pREP constructs were used to transform S. pombe leu1-32 to leu prototrophy (18). Transformants were isolated and grown to early log phase in medium containing 2 μM- thiamine. Cultures were then spun and washed in thiamine-free EMM2 and allowed to resume growth. After 24 hrs. cells were collected and transferred to fresh medium, either EMM2 or EMMP. The latter is selective synthetic medium which contains a low phosphate concentration. After 2hrs, cultures were tested for the translocation of proteins. After harvesting the cultures, cells were separated from the growth medium by centrifugation and directly applied to coverslips. Fluorescence of the (SPL)-GFP expressing cells was visualized without fixative treatment of the cells using a LEITZ fluorescence microscope. For immunoblot detection, proteins present in the growth media were acetone precipitated and cells were lysed using a mixture of lysozyme and novozyme (0,5 mg each/ml in 1.2 M sorbitol/PBS). Protein extracts were applied to sodium dodecyl sulphate-polyacrylamide gel electrophoresis according to Laemmli (19). Heterologous proteins were identified by immunoblotting (Fig. 3 and 4) using specific antibodies and enhanced chemiluminescence detection as previously described (5).

Quantification of Western blots. Protein bands visualized after immuno-detection and ECL were quantified by Imagequant (Molecular Dynamics) of the X-ray film.

Measurement of acid phosphatase activity. Yeast cultures were collected and assayed for phosphatase activity (20). The substrate p-nitrophenylphosphate, 2.25 mg in 0.5ml of 0.1 M sodium acetate (pH 4.2), was added to 1 ml culture growth medium. Reactions were run at 37°C for 10 min and stopped by the addition of 0.12 ml of 25% trichloroacetic acid and 0.6 ml of saturated sodium carbonate. The absorbance was measured at 420 nm and one unit of enzyme activity was defined as that liberating 1 μmol of p-nitrophenol per min.

RESULTS

To examine the translocation of proteins in vivo, we expressed the green fluorescent protein (GFP) and the chimera SPL-GFP (Fig. 1) in *S. pombe.* Cells were grown in synthetic selective medium (EMM2) upon induction of the thiamine repressible promoter nmt1. Synthesis of GFP in *S. pombe* resulted in a strong fluorescence signal. The GFP staining throughout the cell was completely random although some organelles or vacuoles were less or not accessible to GFP (Fig. 2). Fusion of the SPL to GFP resulted in a different staining pattern after induction of protein synthesis. Fluorescence of SPL-GFP could be detected peri-nuclearly and in a vesicular pattern suggesting that GFP was transported to the endoplasmic reticulum, the Golgi apparatus and secretory vesicles that are part of the secretory pathway (Fig. 2).

Next we checked if GFP could be efficiently secreted in the medium. Growth medium and cells were separated and subjected to SDS-PAGE and Western blotting. Cells expressing GFP solely intracellularly synthesized more then 1 mg protein per liter culture. The protein bands visualized by immuno-detection appeared to have the correct size as shown (Fig. 3; lane 7 and 8). No GFP could be detected in the growth medium of these cells (Fig. 3; lane 3 and 4). A different result was obtained when growth medium and cells, expressing SPL-GFP, were tested. The amount of (SPL) GFP in these cells was very low (Fig. 3; lane 5 and 6) and the protein possessed a slighter retarded electrophoretic mobility than the native GFP form due to the presence of the leader peptide. In contrast, high levels of GFP protein with the same molecular weight of the native form were detected in the growth medium (Fig. 3; lane 1 and 2), suggesting that SPL-GFP may be correctly processed during secretion. Image quantification of the X-ray film of the immunoblot revealed secretion up to 80% of the total produced GFP. This is in agree-

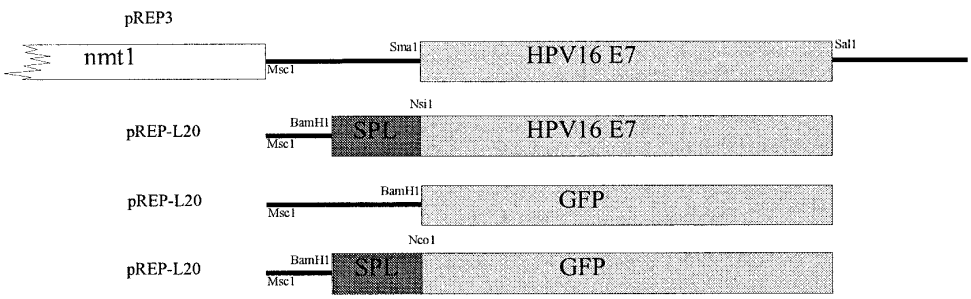


FIG. 1. Vectors were constructed as described in materials and methods. Restriction sites are shown. nmt1, thiamine repressible promoter; SPL, *pho1+ S. pombe* leader sequence.

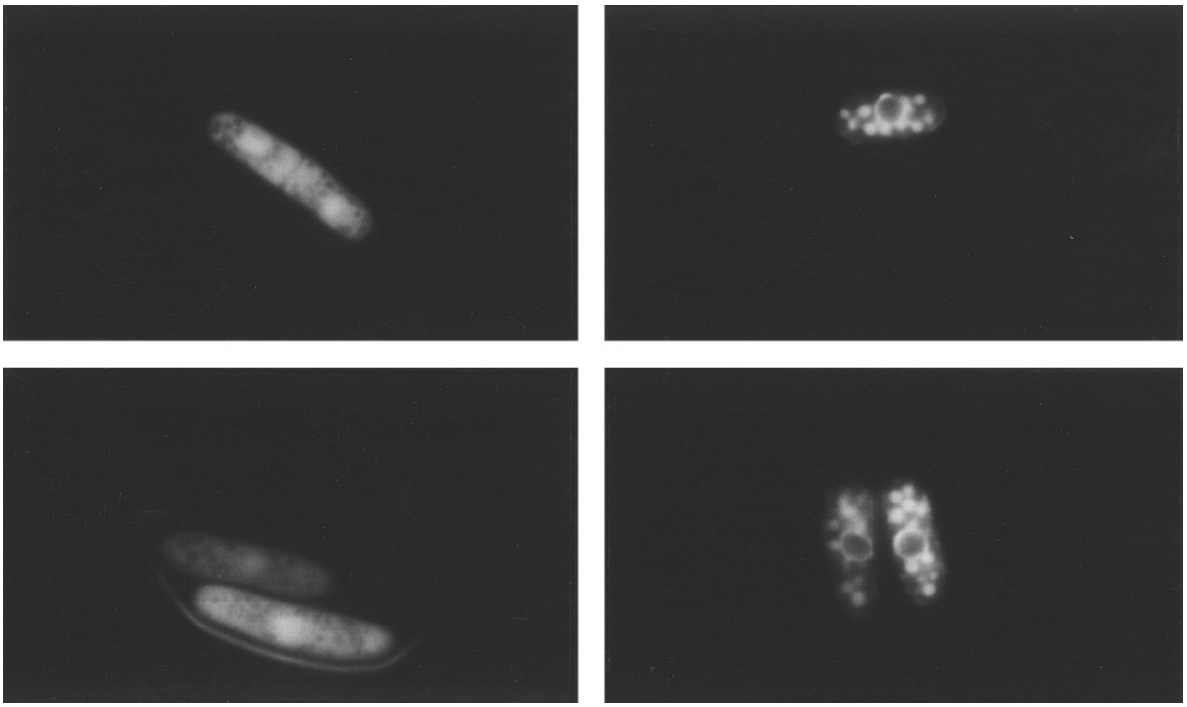
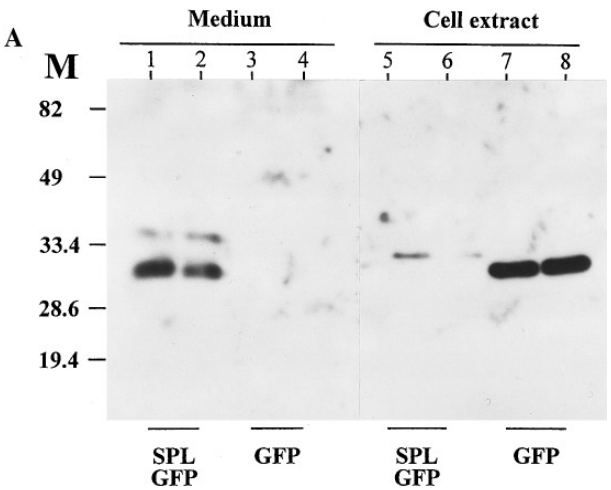


FIG. 2. Fluorescence detection of *S. pombe* cells expressing either GFP or SPL-GFP. *In vivo* detection of GFP in *S. pombe* cells after induction of the thiamine repressible promoter (nmt1) as described in materials and methods.

ment with the minor fraction of intracellular SPL-GFP (Fig. 3; lane 5 and 6) that is still present within the cell and represents around 15 % of the total synthesized GFP. This indicates an efficient secretion system where levels of up to 0.8 mg protein per liter cell culture can be obtained. We also tested if the secretory system could be suitable for the production of recombinant proteins which are normaly targeted to the nucleus. For this purpose we have chosen the oncoprotein HPV 16 E7 which is known to be localized in the nucleus of mammalian and yeast cells. Yeast strains carrying the HPV 16 E7 gene or the SPL-16E7 construct (Fig. 1) were grown in EMM2 upon induction of the thiamine repressible promotor nmt1 (1). Cells were then transferred to fresh EMM2, as described in materials and methods. Cultures were collected and cell number was determined photometrically. Cells and growth medium were separated and equal protein amounts were applied to SDS-PAGE and immunoblotted for the presence of the viral protein. No HPV 16 E7 could be detected within the growth media of cells producing intracellular HPV 16 E7 only (data not shown). However, cells expressing SPL-16E7 secreted a small but clearly detectable amount of E7 protein into the growth medium (data not shown and Fig. 4A lane 4).

It has been previously reported that secretion efficiency of proteins depends on the growth medium conditions in the yeast *S. cerevisae* (21) (22). We have tested if this is also valid for the yeast *S. pombe*. We modified the growth medium and analyzed the cell culture supernatants for an increase of 16 E7 protein secretion. The low secretion efficiency of E7 in EMM2 (Fig. 4A lane 4) can be significantly increased up to 70% in the low phosphate medium, EMMP (Fig. 4A lane 3). Since hardly any differences in the total amount of synthesized E7 protein could be observed after Image quantification it seems that secretion efficiency, using this leader peptide, is related to the phosphate concentration in the growth medium.

In order to investigate whether GFP and HPV 16 E7 are secreted through the same pathway as the native acid phosphatase from which the leader peptide was derived we analyzed if the expression of heterologous proteins interfered with the secretion efficiency of the endogenous phosphatase. We measured the total and secreted amount of phosphatase activity in cells expressing intracellular GFP or 16 E7 compared with cells expressing the leader (SPL) fused polypeptides. The acid phosphatase conversion of the substrate p-nitrophenylphosphate (20) was measured in culture growth medium in presence or absence of cells (total and secreted protein activity respectively). Upon intracellular expression of the two proteins, activity of acid phosphatase in the growth medium was slightly reduced (Fig. 5; compare EMM2 + Th with EMM2 in 16 E7 and GFP). Fusion of these two proteins with SPL however resulted in a strong decrease of extracellular phosphatase activity representing a reduced secretion

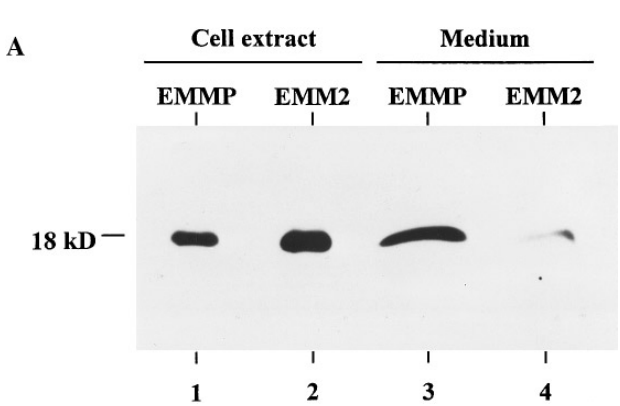


B

Lane	Percent (%)
1	83
2	71
3	1 N.D.
4	1 N.D.
5	23
6	11
7	95
8	100

FIG. 3. **A.** Western blot analysis of the green fluorescent protein. Total cell extract and growth medium of two independent isolated clones were separated by SDS–PAGE and semi-dry blotted onto nitrocellulose as described in materials and methods. M, Molecular weight marker. Lane 1 and 2, the cold acetone-precipitate of supernatant of 5×10^6 pREP-SPLGFP cells. Lane 3 and 4, the cold acetone-precipitate of supernatant of 5×10^6 pREP-GFP cells (intracellular expression). Lane 5 and 6, cell extract of 5×10^6 pREP-SPL-GFP cells. Lane 7 and 8, cell extract of 5×10^6 pREP-GFP cells which express green fluorescent protein intracellular solely. **B.** Image quantification of the immunoblot as shown in Fig.3A. The intracellular produced GFP as detected in lane 8 was set as 100% reference for total GFP synthesis. N.D.: Value not above background.

of this enzyme (Fig. 5; compare GFP or 16 E7 with SPL-GFP or SPL-16 E7). This phenomenon was observed more clearly when cells were transferred to low phosphate medium (EMMP). The amount of secreted acid phosphatase was reduced about three fold in cells expressing SPL-fusion proteins (Fig. 5; compare EMMP + Th with EMMP). This result along with the increase of heterologous protein secretion in low phos-



B

Lane	Percent (%)
1	27
2	97
3	73
4	3

FIG. 4. Secretion of HPV 16 E7 protein is increased in low phosphate medium. **A.** Lane 1 and 2, intracellular expression of 5×10^6 SPL-16 E7 cells in different media. Lane 3 and 4, the cold acetone-precipitate of supernatant of the same amount of SPL-16 E7 cells (lane 1 and 2, respectively). Proteins were separated on a 0.1 % SDS-12.5 % polyacrylamide gel and semi-dry blotted onto nitrocellulose (Schleicher and Schuell, 0.45 μm) and treated as described in materials and methods. **B.** Image quantification of the immunoblot as shown in Fig. 4A. Secreted and intracellular produced HPV 16 E7 protein were normalized to 100 % for the 2 media.

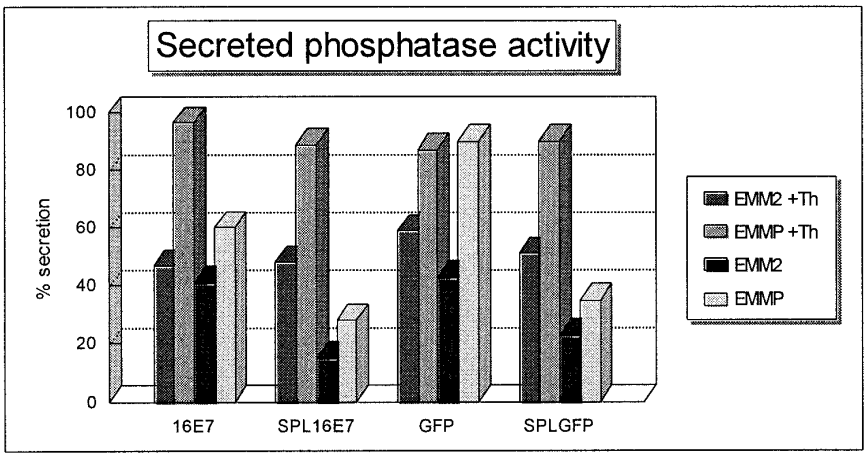


FIG. 5. Influence of recombinant protein expression upon secreted phosphatase activity. Cells were grown as described in materials and methods. Cultures were split equally and cultivated further in presence or absence of the repressor thiamine (Th) either in EMM2 or EMMP. In absence of thiamine, cells expressed the leader (SPL) fused proteins or unfused proteins. Total phosphatase activity of yeast cultures was measured directly or cells were separated from the medium by centrifugation and the phosphatase activity in the supernatant was determined using nitro-phenyl-phosphate as substrate. Relative percentage of secreted were calculated and corrected for the growth medium. Values represent the mean of 5 independent experiments.

phate medium suggest a competition during translocation and therefore a specific secretion of the recombinant proteins.

DISCUSSION

The ability of the *S. pombe* major acid phosphatase leader sequence to secrete heterologous proteins was investigated in this report. Our data presented here argue for specific secretion rather than nonspecific leakage of the recombinant proteins from the yeast cells. First, the cells expressing secretory GFP showed a clearly different appearance in fluorecence microscopy from that of cells expressing intracellular GFP. The peri-nuclear and vesicular staining pattern of GFP within these live cell preparations are in agreement with accumulation of GFP within the endoplasmic reticulum, the Golgi apparatus and secretory vesicles, compartments that are part of the secretory pathway. Second, GFP could be detected by immunoblot analysis only within the growth media of cells carrying the SPL-GFP construct and not within the medium of cells expressing intracellular GFP. In addition, only a minor fraction of the totally produced SPL-GFP was retained within the cells. Third, modification of the growth medium conditions clearly improved the secretion efficiency of the viral oncoprotein HPV 16 E7 which is in agreement with previous studies on the budding yeast *S. cerevisae* (21) (22). Finally, expression of the two recombinant proteins when fused to SPL resulted in a decreased secretion of the native acid phosphatase in contrast to intracellular expression of the unfused proteins. So high level expression of the SPL- fused recombinant proteins seem to interfere with the translocation process of the native acid phosphatase and suggests secretion to occur through the same pathway.

In conclusion, our results indicate that SPL bearing constructs can be applied for the production of recombinant proteins even if a NLS is present within the protein of interest. Further, secretion of recombinant proteins can be improved upon modification of the growth medium as we demonstrated for the HPV 16 E7 protein. In addition, the efficient secretion of GFP makes it a powerful tool to study the secretion pathway of *S. pombe*. It is expected that secretory mutant strains will accumulate GFP at specific steps during the translocation process which can be visualized in vivo and helps to identify specific functions of proteins involved in the secretory pathway of *S. pombe*.

ACKNOWLEDGMENTS

The authors wish to thank Dr. K. Maundrell (Glaxo) for providing the pREP constructs, Dr. M. Pawlita for helpful advice in the early stages of this work and Francesca Venturini for critical reading of the manuscript.

REFERENCES

1. Maundrell, K. (1993) *Gene* **123,** 127–130.
2. Atkins, D., and Izant, J. G. (1995) *Curr. Genet.* **28,** 585–588.
3. Lu, Q., Bauer, J., and Greener, A. (1997) *Gene* **200,** 135–144.
4. Forsburg, S., and Sherman, D. (1997) *Gene* **191,** 191–195.
5. Braspenning, J., Manetti, R., Zumbach, K., Meschede, W., Gissmann, L., and Tommasino, M. (1997) *Protein Expr. Purif.* **10,** 192–201.
6. Elliott, S., Chang, C. W., Schweingruber, M. E., Schaller, J., Rickli, E. E., and Carbon, J. (1986) *J. Biol. Chem.* **261,** 2936–2941.

7. Inouye, S., and Tsuji, F. I. (1994) *FEBS Lett.* **341,** 277–280.
8. Heim, R., Cubitt, A. B., and Tsien, R. Y. (1995) *Nature* **373,** 663–664.
9. Hu, W., and Cheng, C. L. (1995) *FEBS Lett.* **369,** 331–334.
10. Barthmaier, P., and Fyrberg, E. (1995) *Dev. Biol.* **169,** 770–774.
11. Sato, H., Furuno, A., and Yoshiike, K. (1989) *Virology* **168,** 195–199.
12. Tommasino, M., Contorni, M., Scarlato, V., Bugnoli, M., Maundrell, K., and Cavalieri, F. (1990) *Gene* **93,** 265–270.
13. Kanda, T., Zanma, S., Watanabe, S., Furuno, A., and Yoshiike, K. (1991) *Virology* **182,** 723–731.
14. Greenfield, I., Nickerson, J., Penman, S., and Stanley, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11217–11221.
15. Fujikawa, K., Furuse, M., Uwabe, K., Maki, H., and Yoshie, O. (1994) *Virology* **204,** 789–793.
16. Zatsepina, O., Braspenning, J., Robberson, D., Hajibagheri, M. A., Blight, K. J., Ely, S., Hibma, M., Spitkovsky, D., Trendelenburg, M., Crawford, L., and Tommasino, M. (1997) *Oncogene* **14,** 1137–1145.
17. Durst, M., Gissmann, L., Ikenberg, H., and zur Hausen, H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3812–3815.
18. Broker, M. (1993) *Biotechniques* **15,** 598–600.
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
20. Bergman, L. W. (1986) *Mol. Cell Biol.* **6,** 2298–2304.
21. De Nobel, J. G., and Barnett, J. A. (1991) *Yeast* **7,** 313–323.
22. Rossini, D., Porro, D., Brambilla, L., Venturini, M., Ranzi, B., Vanoni, M., and Alberghina, L. (1993) *Yeast* **9,** 77–84.