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E_1BF is an essential RNA polymerase I transcription factor with an intrinsic protein kinase activity that can modulate rRNA gene transcription

(rDNA transcription control/protein phosphorylation)

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ABSTRACT **We previously described the purification and characterization of E_1BF, a rat rRNA gene core promoter-binding factor that consists of two polypeptides of 89 and 79 kDa. When this factor was incubated in the absence of any exogenous protein kinase under conditions optimal for protein phosphorylation, the 79-kDa polypeptide of E_1BF was selectively phosphorylated. The labeled phosphate could be removed from the E_1BF polypeptide by treatment with calf intestinal alkaline phosphatase or potato acid phosphatase. Elution of the protein from the E_1BF–promoter complex formed in an electrophoretic mobility-shift assay followed by incubation of the concentrated eluent with [γ-^{32}P]ATP resulted in the selective labeling of the 79-kDa band. The E_1BF-associated protein kinase did not phosphorylate casein or histone H1. Fraction DE-B, a preparation containing RNA polymerase I and all polymerase I transcription factors (including E_1BF), lost polymerase I transcriptional activity when treated with phosphatase. The phosphatase-induced inactivation of polymerase I activity associated with fraction DE-B could be reversed by the addition of purified E_1BF. Treatment of purified E_1BF with heat, SDS, or an ATP affinity analog eliminated its capacity to reactivate dephosphorylated fraction DE-B. These data demonstrate that (*i*) polymerase I promoter-binding factor E_1BF contains an intrinsic substrate-specific protein kinase and (*ii*) E_1BF is an essential polymerase I transcription factor that can modulate rRNA gene transcription by protein phosphorylation. Further, these studies have provided a direct means to identify a protein kinase or any other enzyme that can interact with a specific DNA sequence.**

Ribosomal RNA represents as much as 80% of total RNA in higher eukaryotes and its synthesis is highly regulated in response to a variety of physiological and pathological stimuli (for reviews, see refs. 1 and 2). Transcription of rRNA genes (rDNA) is directed by RNA polymerase I and other accessory transcription factors. E_1BF is a factor that can interact with an upstream enhancer element (E1) (3, 4) as well as the promoter sequence and can stimulate rat rDNA transcription *in vitro* (5). Recent studies have demonstrated that it can also activate the human rDNA promoter, but not polymerase II promoters (H.N. and S.T.J., unpublished data). When analyzed by SDS/PAGE under reducing conditions, purified E_1BF displays two silver-stained bands of M_r 89,000 and 79,000. However, the mechanism by which E_1BF modulates rDNA transcription has not been elucidated.

Phosphorylation of certain protein factors can modify RNA polymerase II-directed transcription (6–11). Similarly, the functions of growth factor receptors (12) and oncogene products (13) are regulated by phosphorylation. A DNA-activated protein kinase can phosphorylate the polymerase II transcription factor Sp1 during transcription *in vitro*, and phosphorylation of Sp1 *in vivo* and *in vitro* is dependent on its binding to the cognate DNA recognition element (10). Also intriguing is the relationship of the hepatitis B virus-encoded transcriptional trans-activator hbx to a novel serine/threonine protein kinase (14).

In contrast to polymerase II transcription, phosphorylation of a specific factor in the polymerase I-directed transcription of rDNA has not been reported. Several years ago, our laboratory showed that RNA polymerase I can be activated by purified protein kinase NII (15), which can stimulate polymerase I activity in a filter binding assay (16). To date, however, neither we nor others have been able to demonstrate that phosphorylation of RNA polymerase I is essential for rDNA transcription. Preliminary studies suggested to us that E_1BF might be posttranslationally modified. We postulated that this modification might be caused by phosphorylation of the factor. To determine whether E_1BF is regulated by phosphorylation/dephosphorylation and to identify the protein kinase that might regulate polymerase I-directed transcription, we initiated a series of experiments. These studies demonstrated that (*i*) E_1BF can indeed be phosphorylated by a protein kinase intrinsic to this factor, (*ii*) this phosphorylation is confined to the 79-kDa polypeptide of E_1BF, and (*iii*) the phosphatase-induced inactivation of polymerase I transcription can be restored by the addition of purified E_1BF.

METHODS

Purification of E_1BF. E_1BF was purified from rat mammary adenocarcinoma cell extract as described (5). Purification by the oligonucleotide affinity column chromatography was repeated four times to avoid contamination with other polypeptides. The final preparation contained two polypeptides of M_r 89,000 and 79,000 (5).

Phosphorylation of E_1BF *in Vitro*. To achieve maximal phosphorylation, E_1BF was completely dephosphorylated prior to the phosphorylation reaction. Purified E_1BF was suspended in 20 mM Hepes, pH 7.9/20% (vol/vol) glycerol/0.1 mM EDTA/2 mM dithiothreitol/0.5 mM phenylmethanesulfonyl fluoride/5 mM $MgCl_2$ and incubated with 0.5 unit of calf intestinal alkaline phosphatase for 10 min at room temperature before addition of sodium vanadate (0.1 mM) to inhibit the phosphatase activity (17, 18). The phosphorylation reaction was performed by incubating the dephosphorylated E_1BF with [γ-^{32}P]ATP (5 μCi, 3000 Ci/mmol; 1 Ci = 37 GBq) at 37°C for 30 min, and the reaction was terminated by the addition of an equal volume of 30% (wt/vol) trichloroacetic acid for precipitation. The pellet was dissolved in SDS loading buffer and kept in a water bath at 100°C for 3 min. The phosphorylated proteins were separated by SDS/10% PAGE

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(19) and detected by autoradiography. The Rainbow protein molecular weight markers (Amersham) were used to determine the molecular masses of proteins.

Electrophoretic Mobility-Shift Assay and Elution of Specific DNA–Protein Complexes. The synthetic oligonucleotide containing the promoter region (base pairs −36 to +18 relative to the transcription start site) of rat rDNA was labeled at the 3′-end with [α-^{32}P]dATP (3000 Ci/mmol) and Klenow fragment of DNA polymerase I and used as the probe in electrophoretic mobility-shift assays as described (5). The specific DNA–protein complexes were separated in a nondenaturing 8% polyacrylamide gel and detected by autoradiography. The gel slices containing specific DNA–protein complexes were excised and the complexes were eluted overnight at 4°C in 400 μl of 50 mM Tris·HCl, pH 7.9/0.01% bovine serum albumin/150 mM NaCl/0.1 mM EDTA/5 mM dithiothreitol. The eluate was concentrated in a Centricon 10 microconcentrator (Amicon) and used for autophosphorylation assays.

***In Vitro* Transcription.** *In vitro* transcription assays were performed essentially as described (20). The plasmid pB7-2.0, which contains rat rDNA sequence spanning from −167 base pairs to +2.0 kilobases relative to the transcription start site (21), was used in this study. The plasmid was cleaved with *Xho* I and used as a template that must yield a 635-nucleotide run-off transcript if transcription starts at the +1 site (21). ^{32}P-labeled ϕX174 DNA digested with *Hae* III was used as size markers.

RESULTS

E_1BF Can Be Phosphorylated by an Endogenous Protein Kinase. As reported previously (5), purified preparations of E_1BF always contained two polypeptides of M_r 89,000 and 79,000 even after extensive fractionation on the oligonucleotide affinity columns. It was of interest to determine whether these proteins can be posttranslationally modified, particularly by phosphorylation, as a few polymerase II transcription factors have been recently shown to be phosphorylated and such modification could modulate transcription. To address the first issue, purified E_1BF was initially incubated in the absence of any exogenous protein kinase under the phosphorylation condition (see *Methods*). Surprisingly, the 79-kDa polypeptide of E_1BF was phosphorylated by an endogenous protein kinase (Fig. 1). To confirm that the labeling of the 79-kDa polypeptide was indeed due to phosphorylation, calf intestinal alkaline phosphatase (Fig. 2, lane 2) or potato acid phosphatase (lane 3) was added to the samples following the kinase reaction and incubation was continued for 30 min. The phosphatase treatments completely eliminated the phosphate from the 79-kDa polypeptide, as evidenced by the lack of a labeled band in the autoradiogram (Fig. 2). Further, unlike [γ-^{32}P]ATP, [α-^{32}P]ATP radioactivity was not incorporated into the protein, as measured by filter binding assay (data not shown). These data indicate that the 79-kDa entity of E_1BF is a phosphoprotein and that addition of phosphate to this protein is directed by an endogenous protein kinase. Since the 89-kDa polypeptide was not phosphorylated *in vitro*, it is unlikely that the smaller polypeptide is a degradation product of the larger polypeptide. Further, elution of the complex formed between the 89-kDa polypeptide and the promoter fragment did not reveal any protein kinase activity associated with this molecule.

The Specific Promoter–E_1BF Complex Contains an Intrinsic Protein Kinase Activity That Phosphorylates the 79-kDa Polypeptide. To confirm further the authenticity of the protein kinase associated with purified E_1BF, the specific oligonucleotide–E_1BF complex obtained under nondenaturing conditions was eluted with a suitable buffer, concentrated, incubated with [γ-^{32}P]ATP, and subjected to SDS/PAGE.

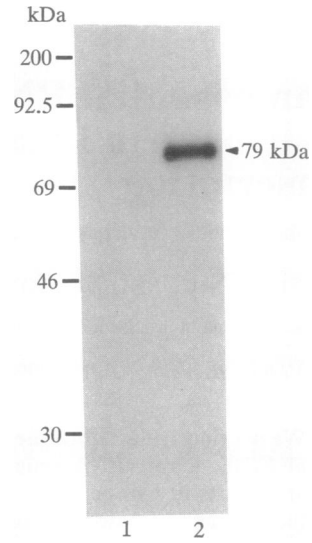

FIG. 1. Phosphorylation of 79-kDa polypeptide by the endogenous kinase activity of E_1BF *in vitro*. The kinase reaction mixture was incubated for 30 min at 37°C. The phosphorylated proteins (labeled with 5 μCi of [γ-^{32}P]ATP) were visualized by electrophoresis in an SDS/10% polyacrylamide gel and autoradiography. Lane 1, kinase reaction in the absence of purified E_1BF; lane 2, reaction with 5 μl of purified E_1BF.

Under the denaturing condition, the labeled free oligonucleotide probe will not be retained in the gel. The only labeled band corresponded to the 79-kDa polypeptide (Fig. 3). Further, the sample eluted from the complex contained the protein kinase activity whereas none of the remaining part of the gel exhibited any protein kinase activity (data not shown). These data have collectively demonstrated that the protein kinase present in the purified preparations of E_1BF is not a minor contaminant undetectable by silver staining and that only the 79-kDa polypeptide acts as the substrate for the protein kinase. Further, we have performed the phosphorylation reaction under several conditions none of which facilitated phosphorylation of the 89-kDa polypeptide associated

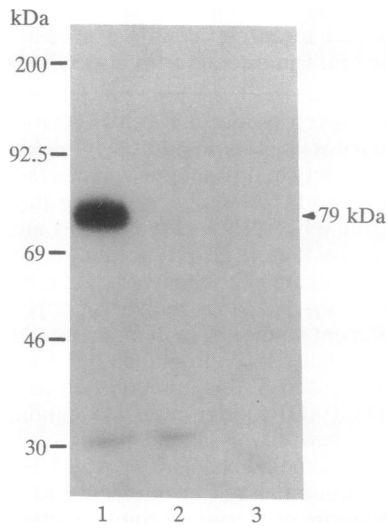

FIG. 2. Effect of calf intestinal alkaline phosphatase and potato acid phosphatase on the phosphorylation of E_1BF *in vitro*. Purified E_1BF (5 μl) was preincubated with 5 μCi of [γ-^{32}P]ATP in the kinase reaction buffer for 10 min at 37°C prior to the addition of calf intestinal alkaline phosphatase or potato acid phosphatase. The sample was then incubated for another 30 min. Lane 1, without any phosphatases; lane 2, with 4 units of calf intestinal alkaline phosphatase; lane 3, with 0.02 unit of potato acid phosphatase.

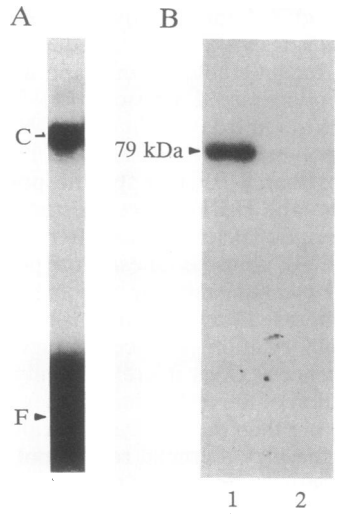

FIG. 3. Phosphorylation of 79-kDa polypeptides following elution of proteins from specific DNA–protein complex formed under nondenaturing conditions. (*A*) Purified E_1BF (20 μl) was incubated with 20 ng of 3′-end-labeled 54-base-pair rat rDNA promoter probe for 60 min at 4°C and loaded onto a nondenaturing 8% polyacrylamide gel for electrophoresis. Positions of the DNA–protein complex (C) and the free probe (F) are indicated at left. (*B*) The DNA–protein complex in *A* was eluted, concentrated, and incubated with (lane 1) or without (lane 2) [γ-^{32}P]ATP under the optimal conditions for phosphorylation (see *Methods*).

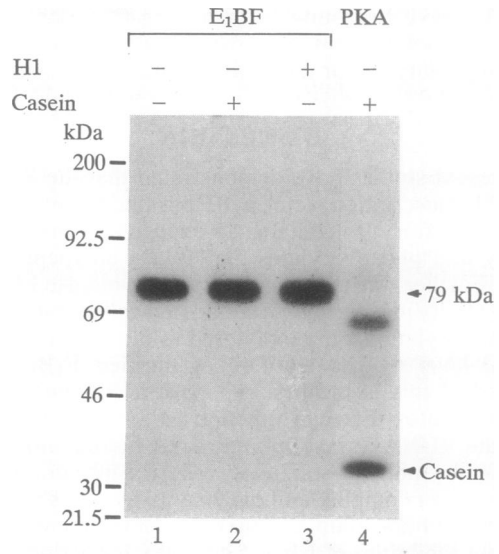

FIG. 4. Substrate specificity of the protein kinase. α-Casein and histone H1 were used along with E_1BF in the protein kinase assay. The phosphorylated proteins were visualized by autoradiography after SDS/10% PAGE. Lane 1, purified E_1BF incubated with [γ-^{32}P]ATP under the standard protein kinase assay conditions; lanes 2 and 3, purified E_1BF incubated with [γ-^{32}P]ATP in the presence of 1 μg of α-casein or histone H1, respectively; lane 4, 1 μg of α-casein incubated with 2 picomolar units of catalytic subunit of protein kinase A (PKA) in the absence of purified E_1BF.

with E_1BF. Preliminary studies have suggested that the protein kinase activity is intrinsic to the 79-kDa polypeptide (data not shown).

Substrate Specificity of E_1BF Kinase Activity. The majority of the protein kinases can phosphorylate either casein or histone H1. Recently, a DNA-activated protein kinase (22) and the transcriptional trans-activator hbx kinase (14) have been shown to phosphorylate casein and histone H1, respectively. We therefore investigated whether the protein kinase associated with E_1BF can phosphorylate these substrates. For this study, α-casein and histone H1 were separately incubated with [γ-^{32}P]ATP and purified E_1BF under the protein kinase assay conditions (Fig. 4). The only phosphorylated band corresponded to the 79-kDa polypeptide. Neither α-casein nor histone H1 was phosphorylated under these conditions. However, the catalytic subunit of bovine cAMP-activated protein kinase (kinase A) could phosphorylate α-casein (the band just below the 79-kDa position appears to be a contaminant in the α-casein preparations phosphorylated by kinase A). These data show that the 79-kDa polypeptide is a specific substrate phosphorylated by the E_1BF-associated protein kinase.

Restoration of the Dephosphorylation-Induced Inactivation of Polymerase I Transcription Activity by Purified E_1BF. To demonstrate that the E_1BF-associated protein kinase required for polymerase I-directed transcription is a functional entity, fraction DE-B, which contains polymerase I and all polymerase I transcription factors (including endogenous E_1BF), was dephosphorylated by potato acid phosphatase, which completely inhibited rDNA transcription. When increasing amounts of exogenous purified E_1BF were added to this functionally inactive fraction, the rDNA transcript began to emerge. At a concentration of 16 ng per 25 μl of fraction DE-B (which contains 35 μg of protein), more than 60% of the original activity was restored (Fig. 5, compare lane 1 with lane 7). The exogenous E_1BF was significantly more efficient in restoring the transcriptional activity of acid phosphatase-treated fraction DE-B than it was in restoring the activity of alkaline phosphatase-treated DE-B. It is likely that unlike acid phosphatase, alkaline phosphatase removes the terminal phosphate of GTP (which forms the first nucleotide in rat rRNA), which might be affecting the initiation of transcription.

The restoration of the transcriptional activity by the addition of E_1BF to the phosphatase-treated fraction DE-B attests to the functional activity of E_1BF in rDNA transcription. In our hands, sodium vanadate can directly affect polymerase I transcription independent of its effect on the phosphatase. We therefore eliminated its use in the transcription assay. Clearly, we have underestimated the potency of E_1BF-associated protein kinase in polymerase I transcription, as it had to overcome the inhibitory effect of the phosphatase before it could actually exert a positive effect on transcrip-

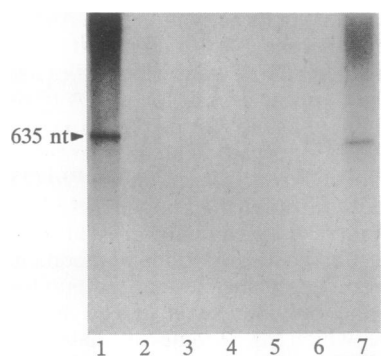

FIG. 5. Restoration by E_1BF of the polymerase I transcriptional activity lost upon dephosphorylation with the phosphatase. *Xho* I-cut plasmid pB7-2.0 (about 50 ng) was used as the template in the reaction. Fraction DE-B (about 35 μg of protein) was preincubated with 0.04 unit of potato acid phosphatase for 10 min at room temperature. Subsequently, purified E_1BF, DNA, and the nucleotide mixture were added and incubated at 30°C for 60 min. The anticipated run-off transcript is 635 nucleotides (nt) long (20). Lane 1, reaction without acid phosphatase and E_1BF; lane 2, reaction in the presence of acid phosphatase without E_1BF; lanes 3–7, reactions in the presence of acid phosphatase with 1, 2, 4, 8, and 16 ng of purified exogenous E_1BF.

tion. We have been unable to restore this activity by the addition of polymerase I or a species-specific factor purified to homogeneity in our laboratory (data not presented).

DISCUSSION

The present studies have demonstrated that the RNA polymerase I transcription factor E_1BF purified and characterized in our laboratory (5) contains an endogenous protein kinase activity that phosphorylates the 79-kDa polypeptide of the factor. Further, the complete loss of polymerase I transcriptional activity in a partially fractionated cell extract following dephosphorylation can be restored to more than 60% of the original level by the addition of purified E_1BF. To our knowledge, this is the first report of a polymerase I transcription factor that can function as a protein kinase and modulate rDNA transcription. DNA-bound enzyme was identified by mobility-shift assay followed by elution from the DNA–protein complex and enzyme assay. As shown previously (5), addition of unlabeled promoter fragment resulted in complete inhibition of labeled complex formation. Further, we used excess labeled DNA for the gel binding assay and the complex formation was proportional to the amount of E_1BF added. The inability of this protein kinase to phosphorylate casein or histone H1 suggests that it is not a general protein kinase. Based on this observation and on its size, it does not appear to be related to any other protein kinases characterized in other laboratories. Heat treatment, denaturation with SDS, and treatment with the ATP affinity analog 5′-(*p*-fluorosulfonylbenzoyl)adenosine, which irreversibly binds to and modifies the ATP-binding domains of protein kinases (23, 24), eliminated the capacity of purified E_1BF to restore activity of the dephosphorylated cell extract (data not shown). These data further prove that the kinase activity associated with E_1BF is essential for its function in rDNA transcription.

We have not established whether only the E_1BF-associated polypeptide is modified by this kinase through autophosphorylation or whether other transcription factors are also phosphorylated under the transcription conditions. Recently, we characterized a polypeptide that can direct species-specific transcription of rat rDNA (unpublished data). This factor is not phosphorylated by the E_1BF kinase (data not shown). Previous studies in our laboratory have shown that RNA polymerase I can be phosphorylated by protein kinase NII *in vitro*, which results in its activation (16). Although phosphorylation of polymerase I can activate its activity (16) and generally enhance rDNA transcription *in vitro*, this modification does not appear crucial for rDNA transcription (J.Z. and S.T.J., unpublished data). There is one report that suggests that phosphorylation of polymerase I is required for the initiation of rDNA transcription *in vitro* (25). Since those studies did not utilize purified polymerase I, it is not known whether polymerase I or a factor present in the polymerase I fraction must be posttranslationally modified for transcriptional initiation. It is plausible that the putative factor associated with the polymerase is indeed the protein kinase reported here, which can become associated with polymerase I as transcription proceeds.

Phosphorylation has been shown to modulate the activities of several polymerase II transcription factors, including the cAMP response element-binding factor CREB (8), the yeast transcriptional activator ADR1 (7), the two octamer motif-binding proteins Oct-1 and Oct-2 (9), the adenovirus E4F transcription factor (26), the GC box-binding factor Sp1 (10), and the hepatitis B virus-encoded transcriptional transactivator hbx (14). Phosphorylation of poly(A) polymerase is essential for the polyadenylylation of pre-mRNAs (K. Chrislip, J.Z., and S.T.J., unpublished data). Here we have described the requirement of a novel protein kinase that can interact with the rDNA promoter sequence and direct its transcription. Recent studies in our laboratory have shown that the rat polymerase I transcription factor E_1BF can augment transcription from the human rDNA promoter, but not from the polymerase II promoters (unpublished data), which indicates that E_1BF is a specific polymerase I transcription factor. The E_1BF-induced restoration of the polymerase I transcriptional activity lost after dephosphorylation indicates that E_1BF kinase is an essential polymerase factor.

The detailed mechanism(s) by which the protein kinase regulates polymerase I transcription has not been elucidated. Does the phosphorylation enhance interaction with specific promoter sequences? Does it preferentially promote initiation vs. elongation of the RNA chain? Does it phosphorylate polypeptides other than the 79-kDa molecule and, if so, does such modification play a crucial role in pol I transcription? Finally, is the activity or level of this protein kinase altered in response to physiological and/or pathological stimuli? Further studies are needed to answer these questions.

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1. Rose, K. M., Stetler, D. A. & Jacob, S. T. (1983) in *Enzymes of Nucleic Acid Synthesis and Modification*, ed. Jacob, S. T. (CRC, Boca Raton, FL), Vol. 2, pp. 43–74.
2. Sollner-Webb, B. & Tower, J. (1986) *Annu. Rev. Biochem.* **55**, 801–830.
3. Dixit, A., Garg, L. C., Chao, W. & Jacob, S. T. (1987) *J. Biol. Chem.* **262**, 11616–11622.
4. Garg, L. C., Dixit, A. & Jacob, S. T. (1989) *J. Biol. Chem.* **264**, 220–224.
5. Zhang, J. & Jacob, S. T. (1990) *Mol. Cell. Biol.* **10**, 5177–5186.
6. Sorger, P. K. & Pelham, H. R. B. (1988) *Cell* **54**, 855–864.
7. Cherry, J. R., Johnson, T. R., Dollaid, C., Shuster, J. R. & Denis, C. L. (1989) *Cell* **56**, 409–419.
8. Gonzalez, G. A. & Montminy, M. R. (1989) *Cell* **59**, 675–680.
9. Tanaka, M. & Herr, W. (1990) *Cell* **60**, 375–386.
10. Jackson, S. P., MacDonald, J. J., Lees-Miller, S. & Tjian, R. (1990) *Cell* **63**, 155–165.
11. Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M. & Hunter, T. (1991) *Cell* **64**, 573–584.
12. Yardeen, Y. & Ullrich, A. (1988) *Annu. Rev. Biochem.* **57**, 443–478.
13. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930.
14. Wu, J. Y., Zhou, Z. Y., Judd, A., Cartwright, C. A. & Robinson, W. S. (1990) *Cell* **63**, 687–695.
15. Rose, K. M., Bell, L. E., Seifken, D. A. & Jacob, S. T. (1981) *J. Biol. Chem.* **256**, 7468–7477.
16. Duceman, B. W., Rose, K. M. & Jacob, S. T. (1981) *J. Biol. Chem.* **256**, 10755–10758.
17. Lopez, U., Stevens, T. & Lindquist, R. N. (1976) *Arch. Biochem. Biophys.* **175**, 31–38.
18. Seargeant, L. E. & Stinson, R. A. (1979) *Biochem. J.* **181**, 247–250.
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
20. Kurl, R. N., Rothblum, L. I. & Jacob, S. T. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6672–6675.
21. Rothblum, L. I., Reddy, R. & Cassidy, B. (1982) *Nucleic Acids Res.* **10**, 7345–7362.
22. Carter, T., Vancurova, I., Sun, I., Lou, W. & Deleon, S. (1990) *Mol. Cell. Biol.* **10**, 6460–6471.
23. Zollner, M. J. & Taylor, S. S. (1979) *J. Biol. Chem.* **254**, 8363–8368.
24. Basu, M., Biswas, R. & Das, M. (1984) *Nature (London)* **311**, 477–480.
25. Schnapp, A., Pfleiderer, C., Rosenbauer, H. & Grummt, I. (1990) *EMBO J.* **9**, 2857–2863.
26. Raychaudhuri, P., Bagchi, S. & Nevins, J. R. (1989) *Genes Dev.* **3**, 620–627.