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Transcription factors important for starting the cell cycle in yeast

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SUMMARY

Unlike early embryonic cleavage divisions in certain animals, cell-cycle progression in yeast and probably also in all metazoan somatic cells requires the periodic transcriptional activation of certain key genes. Thus far, the only clear examples are genes that encode a class of unstable 'cyclin' proteins, which bind and activate the cdc2/Cdc28 protein kinase: the G1-specific cyclins encoded by *CLN1* and *CLN2*, a B-type cyclin implicated in DNA replication encoded by *CLB5*; and four B-type cyclins involved in mitosis encoded by *CLB1*, *2*, *3*, *4*. *CLN1*, *CLN2*, and *CLB5* are transcribed in late G1, as cells undergo Start. A transcription factor composed of Swi4 and Swi6 proteins (called SBF) activates *CLN1* and *CLN2* transcription via a positive feedback loop in which Cln proteins activate their own transcription. A different but related transcription factor called MBF seems responsible for the late G1-specific transcription of most DNA replication genes including *CLB5*. We have purified MBF and shown that it contains Swi6 and a 110–120 kDa protein distinct from Swi4 (p120) that contacts DNA. Thus, we propose that SBF and MBF share a common regulatory subunit (Swi6) but recognize their promoter elements via distinct DNA binding subunits.

1. INTRODUCTION

It was the discovery that DNA replication occurs at a discrete stage during interphase that led to the division of the eukaryotic cell cycle into the now familiar G1, S, G2, and M phases. We now recognize that G1 can be subdivided into early and late phases. Early G1 cells can either embark on a new cell cycle, enter a quiescent state or undergo differentiation. Later in G1, cells become committed to DNA replication and often also to the completion of mitosis. In the budding yeast *S. cerevisiae*, the transition from early to late phases of G1 is known as Start (Pringle & Hartwell 1981). The equivalent process in mammalian cells has been called the restriction point (Pardee 1989). In both yeast and mammalian cells, the execution of Start requires protein synthesis and growth to a critical cell size. Apart from this the Start transition is only poorly understood at a molecular level, raising a number of important questions: What is the physical basis behind Start? What proteins need to be synthesized and what determines the timing of their synthesis? How does their synthesis lead to the onset of S phase?

2. MATERIALS AND METHODS

(*a*) *Gel retardation and DNA crosslinking*

Gel retardation assays were done as previously described (Taba *et al.* 1991). Oligonucleotide probes were from the *TMP1* promoter (MCB-TMP1, Dirick *et al.* 1992; detection of MBF) and from the *CLN2* promoter (pCL2, Nasmyth & Dirick 1991; detection of SBF). DNA crosslinking experiments were carried out in solution using a bromodeoxyuridine-substituted oligonucleotide derived from the *TMP1* promoter as described (Dirick *et al.* 1992; Barberis *et al.* 1990). After crosslinking protein–DNA complexes with UV and digesting them with DNaseI, reaction mixtures were incubated with a 1:100 dilution of polyclonal antiserum for 30 min on ice. Subsequently 5 ml of Protein A sepharose beads (Pharmacia; 50% (by volume) suspension in $N_{50}A_{50}$, preadsorbed with 5 mg ml^{-1} BSA) were added and the mixture incubated for another 30 min at 4°C with slight agitation. The Protein A sepharose beads were then recovered by centrifugation and washed extensively with $N_{50}A_{50}$ buffer (20 mM Tris-HCl pH = 7.5, 50 mM NaCl, 50 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol (by volume)) before SDS-PAGE and autoradiography.

(*b*) *Purification of p120*

The purification procedure employed was basically as described in Sorger *et al.* (1989). A 1000 l culture of a protease-deficient yeast strain (BJ2168; *Mata*, *leu2*, *trp1*, *gal2*, *prb*, *prc*, *pep4*) was grown in mid-exponential phase. The cells were harvested by centrifugation and stored at −80°C until used. Preparing whole-cell

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extract and heparin-agarose chromatography was carried out exactly as described (Sorger *et al.* 1989).

Extracts were usually prepared from 500–600 g of cells, yielding 400–450 ml of 20–25 mg ml^{-1} whole cell extract, which was then loaded onto a 400 ml heparin-agarose column by gravitational flow. The column was washed O/N with approximately 5 volumes of binding buffer (A_{50}, see Sorger *et al.* 1989) and bound proteins were eluted at 2 ml min^{-1} using a linear salt gradient (A_{50}–A_{600}, 4.5 column volumes). The eluted fractions were assayed for MBF activity by gel retardation assays and anti-Swi6 Western blots (Haid & Suissa 1983). Fractions containing the peak of MBF activity were then dialysed against $A_{50}N_{50}$ supplemented with 1 mM DTT and protease inhibitors (0.2 mM PMSF, 50 μM TPCK, 25 μM TLCK, 2 μg ml^{-1} pepstatin). Approximately 40–50 ml of pooled dialysed heparin-agarose column eluates were then loaded onto an 8 ml specific DNA-sepharose column in the presence of 0.1 mg ml^{-1} sonicated salmon sperm DNA and at a flow rate of 0.3 ml min^{-1}. The column was washed O/N with $A_{50}N_{150}$ (as $A_{50}N_{50}$ but 150 mM NaCl) and bound protein was eluted with 2 column volumes of $A_{50}N_{500}$ (as $A_{50}N_{50}$ but 500 mM NaCl). One millilitre fractions were collected and assayed for p120 and Swi6 by anti-Swi6 Western blotting and silver staining. Peak fractions from 4–5 successive runs over the DNA-sepharose column (20–25 ml) were pooled and the protein TCA-precipitated (7.2% trichloroacetic acid, 0.015% Na-deoxycholate; 1 h on ice) in the presence of 2 mg ml^{-1} insulin as a carrier. After washing with cold (−20°C) acetone, the pellets were resuspended in a small volume of sample buffer (Lämmli 1970), boiled and separated by SDS-PAGE. A gel slice containing p120 was then excised from the appropriate part of the gel (i.e. within any staining) and the protein eluted O/N in 50 mM Tris-HCl pH = 7.5, 150 mM NaCl, 0.05% SDS, 0.1 mM EDTA.

The DNA sepharose column was prepared as described (Kadanoga & Tjian 1986; Sorger *et al.* 1989). Synthetic oligonucleotides (700 μg each) derived from the TMP1 promoter (5′-agctTGGAA**ACGCGT**CAATTAAGGTCTTTTTCATTTTTTCTATTTA**ACGCGT**CA-3′; 5′-agctTG**ACGCGT**TAAATAGAAAAAATGAAAAAGACCTTAATTG**ACGCGT**TTCA-3′) were phosphorylated, annealed and ligated to form concatemers consisting of 2–6 monomers. These molecules were coupled to 8 ml of CNBr-activated Sepharose CL-4B (Pharmacia). At a coupling efficiency of 60–70% the amount of coupled DNA was estimated at approximately 60 μg ml^{-1} Sepharose resin.

(c) *Immunodepletion of heparin-agarose fractions*

Five millilitres of heparin-agarose eluate with high MBF activity were treated exactly as described above, except that after dialysis the eluate was split into 2 ml × 2 ml and one half was incubated with 100 μl of SW16 IgGs covalently coupled to Protein A sepharose (50% by volume; 2 mg IgG per millilitre swollen beads) and the other with control IgGs for 30 min at 4°C. The supernatants were then fractionated over a 1 ml DNA affinity column as described above and 1 ml fractions collected.

3. RESULTS AND DISCUSSION

(a) *Start involves the activation of the Cdc28 protein kinase by G1 cyclins*

The analysis of temperature-sensitive mutants that arrest at specific stages of the cell cycle identified 50 or more cell division cycle (*CDC*) genes. Of these, only *CDC28* (Reid & Hartwell 1977) and *CDC37* (Reed 1980) are required for Start. CDC28 encodes a highly conserved protein kinase, which is homologous to the cdc2 kinase of fission yeast and mammalian cells (Lorincz & Reed 1984). The function of *CDC37* remains unknown. One of the striking aspects of the cdc2/CDC28 kinase is that it is required not only for Start but also for mitosis, at least in fungi. Slightly different isoforms of the kinase, cdk2 and cdc2, seem to perform the two tasks in mammalian cells (Pines 1992).

Insight into how the cdc2/Cdc28 kinase can be involved in such diverse functions as DNA replication and mitosis originated with the discovery that the kinase is only active when complexed with a class of proteins called cyclins (Solomon *et al.* 1990). There are many different types of cyclins which have been discovered by diverse means. A and B type cyclins were discovered due to cell-cycle-dependent oscillations in their abundance during sea urchin cleavage divisions (Evans *et al.* 1983), whereas the Cln3 cyclin was discovered because mutants that stabilize it cause yeast cells to undergo Start with an abnormally small cell size (Carter & Sudbury 1980; Nash *et al.* 1988). All cyclins share a conserved domain of 120 amino acids. Most are unstable and most also oscillate in abundance during the cell cycle. The genetic analysis of cyclin function is most advanced in the yeast *Saccharomyces cerevisiae*, where one of three G1-specific cyclins encoded by *CLN1*, *2*, and *3* is required for Start (Richardson *et al.* 1989). B-type cyclins encoded by *CLB1*, *2*, *3*, and *4* are involved in the formation and function of the mitotic spindle (Surana *et al.* 1991), and another B-type cyclin encoded by *CLB5* is necessary for S phase progression (see figure 1). Transcription of all these cyclin genes, apart from CLN3, is cell cycle regulated: *CLN1*, *CLN2*, and *CLB5* transcripts appear transiently as cells undergo Start, *CLB3* and *CLB4* transcripts appear during S phase, and *CLB1* and *CLB2* transcripts appears during G2 (figure 1).

Cdc28 can be isolated as an active kinase when associated with Cln (Wittenberg *et al.* 1990) or Clb cyclins (Amon *et al.* 1992). The Cln1 and Cln2 associated kinase is activated when cells undergo Start and disappears when they enter G2 (Tyers *et al.* submitted), whereas Clb2 associated kinase appears during G2, peaks just prior to anaphase, and is destroyed as cells enter G1 (Surana *et al.* 1993). Kinase activities associated with Clb1, 3, 4, and 5 have not yet been characterized. It is currently

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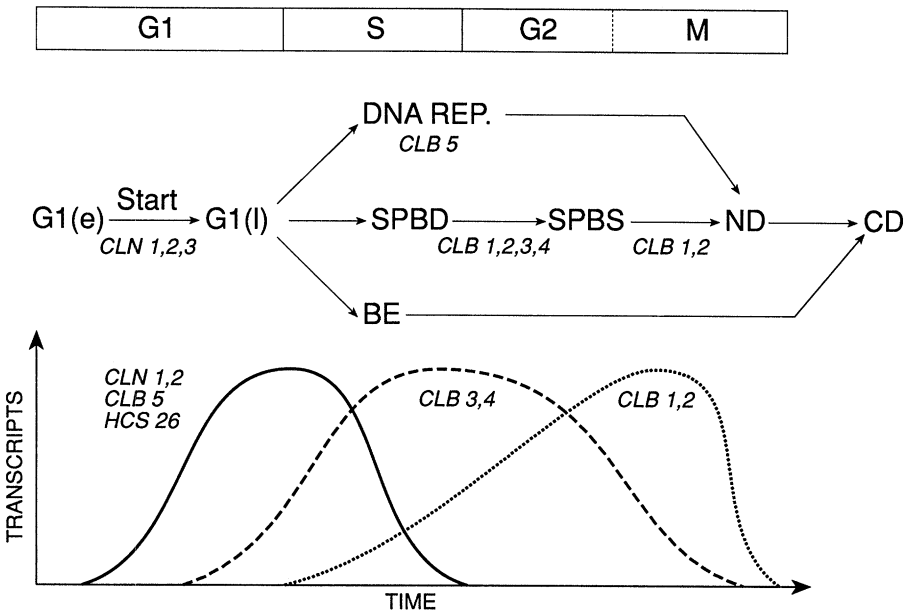

Figure 1. The function and transcriptional regulation of different cyclin genes in *S. cerevisiae*. The G1 period is divided into early and late phases. Preparations from all aspects of the cell division process occur soon after cells undergo Start: cells enter S phase, they duplicate their spindle pole bodies (the first step to forming a mitotic spindle) (Byers 1981), and lay down proteins required for cytokinesis in the vicinity of the future bud site (Kim *et al.* 1991). SPBD and SPDS stand for spindle pole body duplication and separation respectively. BE stands for bud emergence. ND and CD stand for nuclear and cell division respectively. One of *CLN1*, *CLN2*, and *CLN3* is needed for Start. *CLB5* is needed for efficient S phase progression (Epstein & Cross 1992; Schwob & Nasmyth 1993). *HCS26* encodes a cyclin-like protein expressed at the same time as *CLN1*, *CLN2*, and *CLB5*. Increased *HCS26* gene dosage partially rescues the lethality of *swi4* mutants in diploid cells (Ogas *et al.* 1991). Its function at Start is not yet known.

thought that the appearance of a Cln/Cdc28 kinase triggers cells to undergo Start (Tyers *et al.* 1993), whereas the appearance of Clb/Cdc28 kinase triggers the onset of mitosis (Fitch *et al.* 1992), and that the destruction of Clb/Cdc28 kinase due to proteolysis of its cyclin subunit triggers the exit from mitosis and re-entry into G1(Surana *et al.* submitted).

Transcriptional regulation of *CLN1* and *CLN2* plays an important, if not vital, part in the timing of Start during yeast proliferation. Both transcripts are absent in small early G1 cells but appear suddenly around the time of Start (Nasmyth & Dirick 1991; Price *et al.* 1991). Moreover, unscheduled expression of either gene from the *GAL* promoter seems to accelerate the entry of cells into S phase (Tyers *et al.* 1993). What leads to the sudden activation of *CLN1* and *CLN2* in late G1? What is known about transcription factors whose activity may be specific to late G1 cells?

(*b*) *G1 cyclin genes are activated by the Swi4/Swi6 transcription factor*

Swi4 and Swi6 were discovered as transcription factors needed for the activation of the *HO* gene, which encodes an endonuclease that induces mating type switching (Nasmyth & Shore 1987; Herskowitz 1989). *HO* was the first example of a large family of genes, including those for G1 cyclins and most DNA replication enzymes, that are activated at Start (Nasmyth 1983). Activation of this gene family is dependent on the Cdc28 protein kinase but is not dependent on other genes required for entry into S phase. An important reason for studying *HO* regulation was the hope that it would prove to be a useful tool for investigating the function of *CDC28*; i.e. *HO* activation seemed a good biochemical marker for Start.

The singling out of Swi4 and Swi6 as factors specifically concerned with a *CDC28* or Start dependent activation pathway relied on the prior identification of a sequence element, now known as the SCB (Swi Cell cycle Box), that is sufficient for conferring this aspect of *HO* regulation (Nasmyth 1985). Deletion of the region of the *HO* promoter containing SCB_s causes *HO* expression during G1 to become simultaneously independent of *CDC28*, *SW14* and *SW16* without affecting the dependence on all eight other SWI genes. In addition, the transcriptional activation of a reporter gene by SCB elements alone was shown to be dependent on *CDC28*, *SW14*, and *SW16* but not on the other SWI genes (Breeden & Nasmyth 1987*a*). We now know that Swi4 and Swi6 are two components of a factor called SBF (SCB Binding Factor) that binds to multiple SCB elements (Andrews & Herskowitz 1989*a*; Taba *et al.* 1991). SBF can be detected in crude yeast extracts using a gel retardation assay (Andrews & Herskowitz 1989*b*; Taba *et al.* 1991) and it can be re-constituted by co-translation of Swi4 and Swi6 in a reticulocyte lysate (Primig *et al.* 1992). The SCB is recognized by a 120 amino acid DNA binding domain at the N-terminus of the Swi4 protein. Unlike Swi4, Swi6 cannot bind SCB DNA on its own in vitro but it can be recruited

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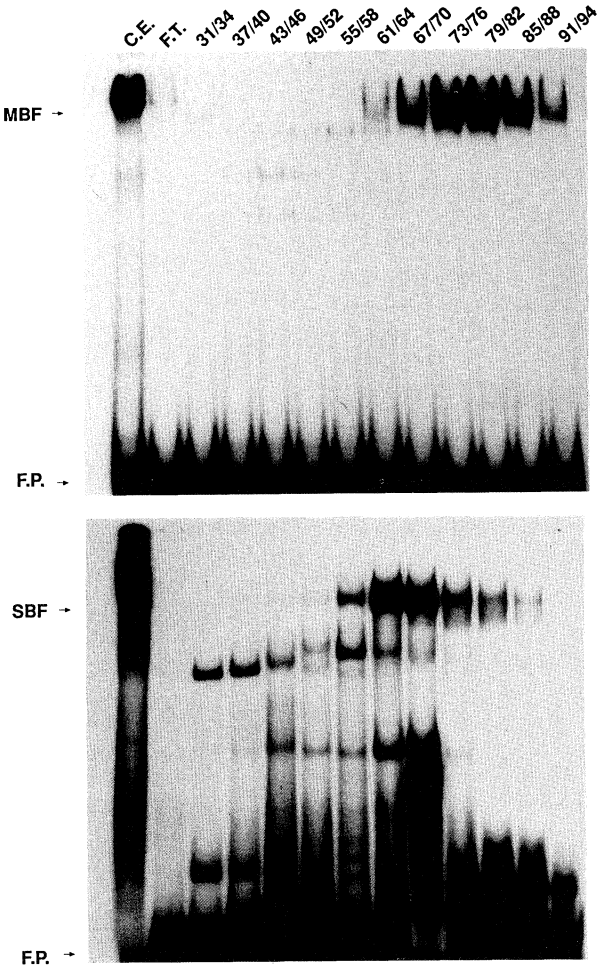

Figure 2. Heparin fractionation of yeast extracts. Yeast whole-cell extracts were prepared and fractionated over a heparin agarose column as described in Materials and Methods. Gel retardation assays were performed on crude extracts (c.e., 2 µl), the flow through (f.t., 2 µl), as well as fractions eluted from the column using a 50–600 mM $(NH_4)_2SO_4$ gradient. Aliquots from two nearby fractions (2 µl) were pooled as indicated and mixed with 0.5 ng of oligonucleotide (corresponding to 10^4 c.p.m.) for each DNA binding assay. MBF (top panel) and SBF (bottom panel) were assayed using probes derived from the *TMP1* and the *CLN2* promoter respectively. The volumes of crude extract (25 mg ml^{-1} protein) and of fractions containing high levels of MBF (0.2 mg ml^{-1} protein) were both approximately 400 ml. We therefore estimate a 40–50% recovery with a 50–60-fold purification. The free probe is labelled with F.P.

into ternary complexes due to the interaction of Swi4 and Swi6 via their C-terminal sequences (Priming *et al.* 1992).

Although neither *SWI4* nor *SWI6* are essential genes in haploid yeast strains, the deletion of both genes causes lethality, implying that they have functions in addition to the activation of *HO* (Breeden & Nasmyth 1987*a*). It is now clear that one of these functions is the activation of the G1 cyclin genes *CLN1* and *CLN2* (Nasmyth & Dirick 1991, Ogas *et al.* 1991). Both genes are poorly transcribed in *swi4* or *swi6* mutants and their expression from a moderately active foreign promoter is sufficient to rescue the double mutant (Nasmyth & Dirick 1991).

(*c*) *Start may involve a positive feedback loop*

The discovery that SBF is required for the activation of *CLN1* and *CLN2* brought to light an important paradox. SBF activity at *HO* needs an active CDC28 kinase, which in turn requires the expression of *CLN1* and *CLN2*; i.e. SBF is not only dependent on the Cdc28 kinase but also an activator of it. There are two explanations for this paradox: either there are two modes of action of SBF, one that is *CDC28*-independent and involved in the activation of G1 cyclins and a second that is *CDC28*-dependent and involved in the activation of *HO*, or SBF and G1 cyclin activation occurs via a positive feedback loop through which the kinase activates SBF, which activates G1 cyclin transcription, which closes the loop by activating the kinase. As predicted by the latter hypothesis, *CLN1* and *CLN2* transcription is dependent on *CDC28* and on *G1* cyclin activity and can be triggered by ectopic expression of any one G1 cyclin gene (Dirick & Nasmyth 1991; Cross & Tinkelenberg 1991). In its simplest form the positive feedback would involve the activation of SBF by phosphorylation of Swi4 or Swi6 by the Cdc28 kinase. This property has not yet been demonstrated.

A positive feedback loop may help explain the apparent irreversibility of Start. It is to be expected that the SBF/G1 cyclin regulatory circuit would have only two stable states: one with low kinase and a second with high kinase. It seems likely that the transition from the low to the high kinase state forms the biochemical basis to Start. This event normally only occurs when cells reach a critical cell size. Such a property ensures the coordination between cell division and growth but how it is achieved is not yet understood. A good guess is that there are weak mechanisms for cyclin activation that are not dependent on the pre-existence of kinase activity and that the strength of this activation pathway is somehow proportional to cell size or to the cell's protein synthetic capacity. The Cln3 cyclin, which does not oscillate much during the cell cyclin (Tyers *et al.* 1993), could have an important role in this process. Another key question concerns how G1 cyclin transcription is later repressed as cells enter G2.

(*d*) *A pair of Start-dependent transcription factors*

The group of genes regulated by SBF are not alone in being activated in late G1. Most if not all genes encoding enzymes involved in DNA replication are also transcribed only transiently during the cell cycle, as cells undergo Start (reviewed in Andrews & Herskowitz 1990). The promoters of these genes all contain one or more copies of a sequence motif whose core is an MluI restriction site (Pizzagalli *et al.* 1988). In several cases, these MluI sequences have been shown to be both necessary for the activity of promoters containing them and sufficient to confer cell cycle regulation on a reporter gene (McIntosh *et al.*

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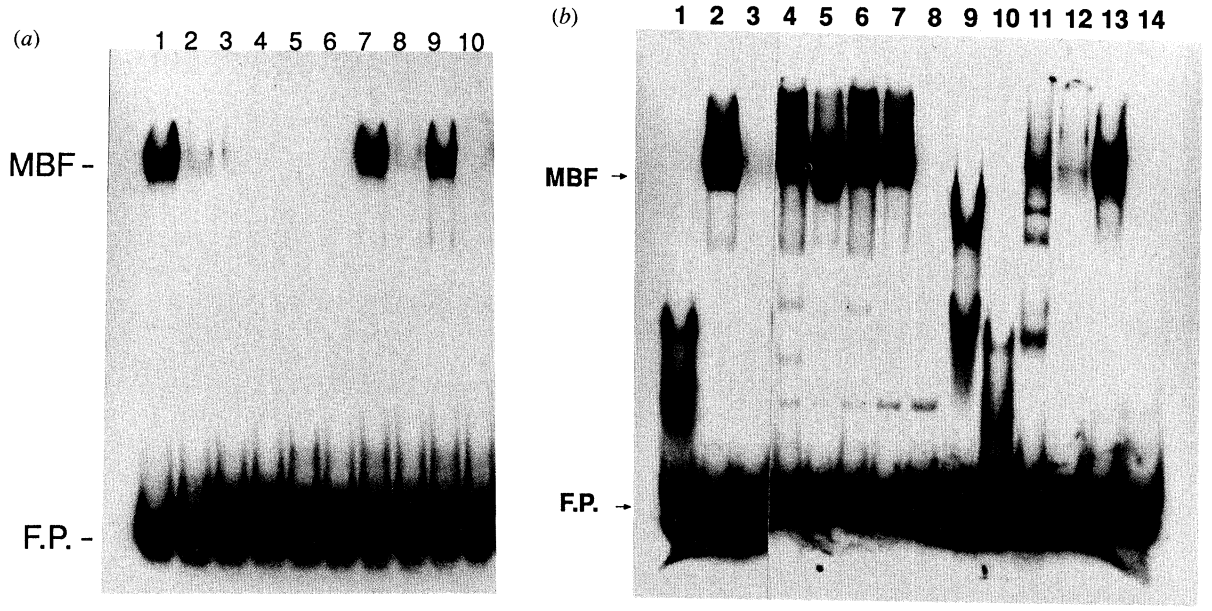

Figure 3. (*a*) MBF activity can be reconstituted after DNA affinity chromatography. Both untreated heparin agarose eluates containing MBF activity (lanes 1, 3 and 5) as well as eluates pre-incubated with anti-Swi6 antibodies coupled to sepharose beads (lanes 2, 4 and 6) were fractionated over a specific MCB-containing DNA-affinity column. Gel retardation assays were performed on 2 µl of both untreated (lane 1) and anti-Swi6 treated (lane 2) heparin agarose eluates, the respective flow throughs (lanes 3 and 4) and the DNA column eluates (lanes 5 and 6). MBF activity is reconstituted upon mixing untreated DNA column eluates with either untreated or anti-Swi6 treated flow through (lanes 7 and 9, 2 µl each) during the DNA binding reaction, but not when mixing immuno-depleted DNA column eluates with the respective flow throughs (lanes 8 and 10, 2 µl each). More than 70% of the MBF activity could be recovered in the eluates after DNA chromatography, as estimated from the mixing experiments. DNA chromatography therefore gives a 200–300-fold purification of MBF. The free probe is labelled with F.P. (*b*) Material eluted from the DNA affinity column was assayed for MBF activity in the presence of various crude protein preparations. Lane 1, eluate only; lane 2, eluate preincubated with flow through of DNA column; lane 3, flow through assayed in the absence of eluate; lane 4, eluate preincubated with 50 µg reticulocyte lysate; lane 5, same as lane 4 but reticulocyte lysate was heated to 75°C for 8 min Before preincubation with eluate; lanes 6 and 7, as in lane 4; lane 8, reticulocyte lysate assayed in the absence of column eluate; lane 9, eluate preincubated with 30 µg BSA; lane 10, eluate preincubated with 20 µg insulin; lane 11, preincubation with 20 µg *E. coli* extract; lane 12, as in lane 11 but after boiling the *E. coli* extract for 15 min; lane 13, 40 µg crude yeast extract was heated to 90°C for 10 min and then preincubated with column eluate; lane 14, heat treated yeast extract assayed in the absence of eluate.

1991; Gordon & Campbell 1991). The motif is therefore now known as the MCB (MluI Cell cycle Box).

We have recently described an activity (called MBF) that binds cooperatively to a pair of MCB elements within the *TMP1* (thymidylate synthase) promoter (Dirick *et al.* 1992). MBF may be the same as an activity called DSC1 (Lowndes *et al.* 1991) that binds to tandemized MluI sites. MBF activity is lacking in *swi6* mutants. Furthermore, gel retardation complexes formed between MBF and *TMP1* promoter DNA are altered in their electrophoretic mobility when treated with Swi6-specific antibodies. Swi6 is therefore at least one component of MBF. UV cross-linking studies indicate that a protein with a molecular mass between 110 and 120 kDa contacts DNA in MCB:MBF complexes. This protein is neither Swi4 nor Swi6 and it has therefore been proposed that MBF, like SBF, is composed of at least two proteins, one of which is Swi6 and the other a novel 120 kDa protein (p120); Dirick *et al.* 1992). Surprisingly, there is almost no change in the abundance of MCB regulated transcripts due to the deletion of *SW16* but there is a profound change

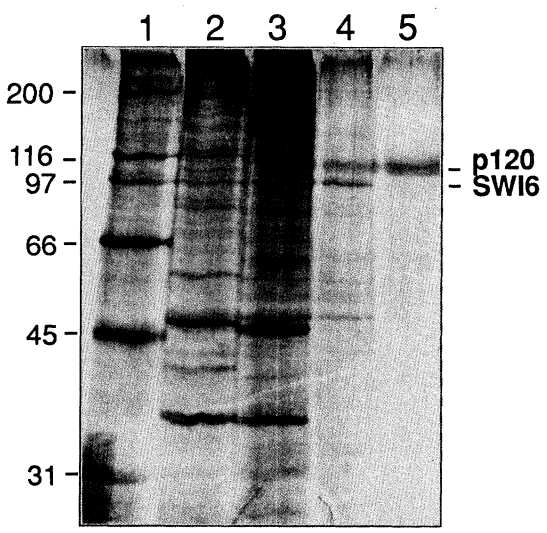

Figure 4. Purification of p120. Proteins present in whole cell extracts (lane 2), after heparin agarose chromatography (lane 3), DNA affinity chromatography (lane 4) and gel purification (lane 5) were visualized by silver staining. p120 and Swi6 are outlined to the right. Molecular mass markers in kDa are shown to the left (lane 1).

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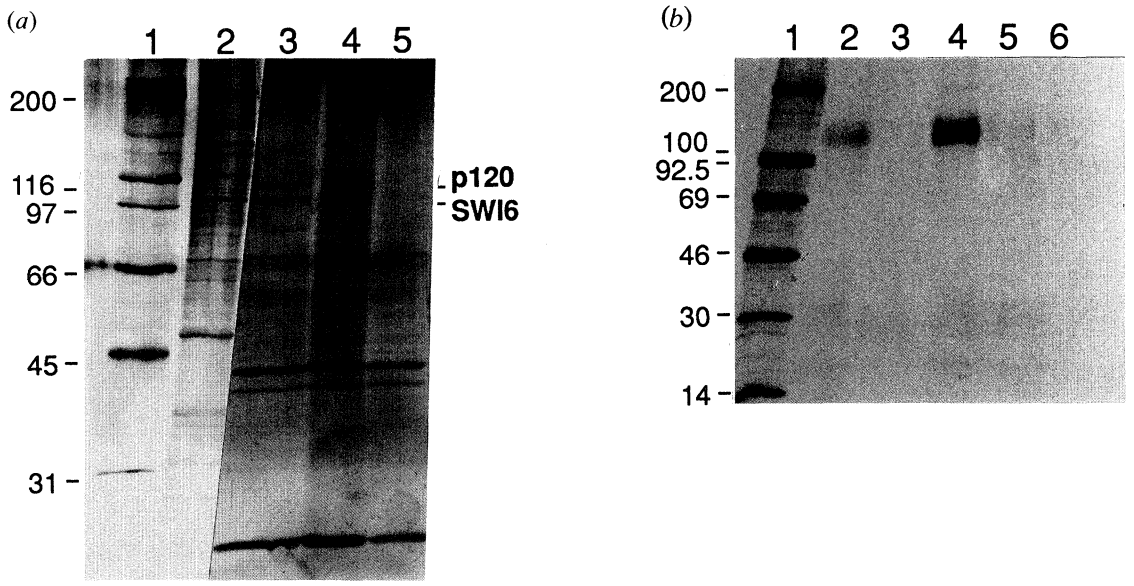

Figure 5. p120 is retained on a specific DNA affinity column. (*a*) Silver staining of proteins. Lane 1: molecular mass markers (in kDa). Lane 2: eluates from heparin agarose column containing the peak of MBF activity. Lanes 3 and 4: eluates from DNA affinity column (eluates from two separate column runs are shown). Lane 5: eluates from DNA affinity column after immunodepletion of heparin agarose eluates with Swi6-specific antibodies. (*b*) Detection of p120 by DNA-crosslinking and co-immunoprecipitation. After incubating radiolabelled MCB containing oligonucleotides (a bromodexyuridine-substituted MCB-TMP1 oligonucleotide; see Dirick *et al.* (1992) for experimental details) with heparin agarose eluates containing MBF activity, protein–DNA complexes were covalently crosslinked by uv-irradiation. Cross-linked complexes were immunoprecipitated with Swi6-specific (lanes 2–4), Swi4-specific (lane 5) or DHFR-specific (lane 6) antibodies after digestion with DNaseI. Either no competitor (lanes 2, 5, and 6), a 50-fold molar excess unlabelled MCB-TMP1 (lane 3), or a 50-fold molar excess of unlabelled point-mutated MCB-TMP1 (both of whose MCBs are mutated, lane 4) were added during the binding reactions.

in their regulation; instead of a sharp peak in transcript abundance in late G1, the transcripts are equally abundant throughout the cell cycle (Dirick *et al.* 1992; Lowndes *et al.* 1992*b*). To characterize further the constituents of MBF, we set out to purify it.

(*e*) *Purification of MBF*

MBF is quantitatively bound to a heparin agarose column matrix and can be eluted from the column using a linear $(NH_4)_2SO_4$ gradient (figure 2; top panel). It was released in an active form between 250 and 350 mM salt (fractions 67 to 88). Western blotting using Swi6-specific antibodies as well as DNA crosslinking experiments confirmed that the eluted MBF peak coincided with a peak of Swi6 protein and contained p120 (data not shown for Swi6, but see figure 5 for the crosslinking). The same set of fractions were assayed for SBF using a DNA probe containing SCBs. SBF was eluted at a slightly lower salt concentration than MBF, confirming that the two factors are distinct.

Eluates from the heparin agarose column containing MBF were dialysed and loaded onto a specific DNA sepharose column (see Materials and Methods). After extensive washing, bound protein was eluted in a single step with 500 mM NaCl. Western blotting confirmed that more than 50% of the Swi6 protein present in the heparin fractions was retained on the DNA column and could be eluted with high salt (data not shown). Surprisingly, we could not detect DNA binding activity in either the flow through or the high salt eluates after DNA sepharose chromatography (figure 3*a*, lanes 3 and 5). Thus, MBF is retained by the DNA column but cannot be recovered in an active form. However, almost all MBF DNA binding activity could be restored by mixing the flow through (which contained more than 95% of the total protein but little or no MBF) with the high salt eluate. This raises the possibility that the MBF complex is labile or that an unstable component of the factor is lost during passage over the DNA sepharose column. Alternatively, protein–DNA interactions may simply be unstable in the very dilute DNA sepharose eluates (2–5 $\mu g\ ml^{-1}$ protein).

To test the latter hypothesis, gel retardation assays with DNA sepharose eluates containing the peak of Swi6 protein were performed in the presence of reticulocyte lysate and *E. coli* whole cell extracts (figure 3*b*, lanes 4–8, 11 and 12). Both protein extracts restored MBF DNA binding, even when heat inactivated. We also observe that also a heat treated yeast crude extract, whose MBF DNA binding activity has been destroyed (figure 3*b*, lane 14), is capable of restoring MBF activity to DNA sepharose eluates (lane 13). These findings suggest that the MBF DNA binding activity detected upon mixing DNA sepharose flow through and high salt eluates is not due to a reconstitution of the MBF transcription factor com-

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plex but is instead due to the creation of an environment which allows DNA binding under the *in vitro* gel retardation reaction conditions.

Figure 4 compares the proteins in the crude extract with those in the heparin agarose and DNA sepharose eluates. Two proteins are prominent in the DNA sepharose column eluate fractions (containing the most Swi6 protein): one migrating at 95 kDa, which corresponds to Swi6, and a second one (marked p120) migrating at 115 kDa, which could correspond to the protein of approximately 120 kDa (p120) previously detected by UV cross-linking experiments (see figure 5*b*). To address whether the 115 kDa protein really corresponds to p120, we analysed the effects of immuno-depleting MBF from heparin-agarose eluates. As shown in figure 5*b*, DNA-crosslinked p120 can be immunoprecipitated using Swi6-specific antibodies (lane 4). It should therefore be possible to remove p120 together with Swi6 from heparin agarose eluates by incubating these fractions with Swi6-specific antibodies and protein A sepharose beads. Figure 3*a* (compare lanes 1 and 2) shows that immuno-depletion using Swi6-specific antibodies leads to an almost complete loss of MBF DNA binding activity. When such depleted heparin agarose fractions were subjected to DNA sepharose chromatography and the high salt eluates were analysed by silver staining, we found that both Swi6 and the 115 kDa protein were selectively removed (figure 5*a*, lanes 4 and 5). Thus, the 115 kDa protein purified along with Swi6 by DNA sepharose chromatography must be associated with Swi6 and is therefore most likely identical to p120. The immuno-depletion experiment also indicates that none of the other polypeptides detected after DNA sepharose chromatography are components of MBF.

As summarized in figure 4, we estimate that we have purified p120 more than 10 000-fold after DNA sepharose chromatography. Preparative SDS-PAGE gel electrophoresis has then used to separate it from Swi6 and other contaminating proteins (figure 4, lane 5). Approximately 25 μg of p120 has been purified, and we are currently trying to obtain antibodies and to determine peptide sequences with the aim of cloning the p120 gene.

(*f*) *MBF might regulate a new class of B-type cyclins*

Unlike SBF, which activates cyclin genes needed for Start, the role of MCB/MBF-directed transcription for cell cycle progression is unclear. Most of the genes regulated by MCB elements encode stable DNA replication enzymes that do not need to be synthesized immediately prior to S phase. This does not exclude the possibility that MBF might also regulate genes which encode unstable proteins needed for S phase. In the course of sequencing the *CLB2* locus, we have discovered that the gene immediately downstream to *CLB2* encodes a new B-type cyclin called *CLB5*. The same gene has also been isolated by Epstein & Cross (1992) by virtue of its ability to complement a *cln1 cln2 cln3* triple mutant. *CLB5* transcripts are cell cycle

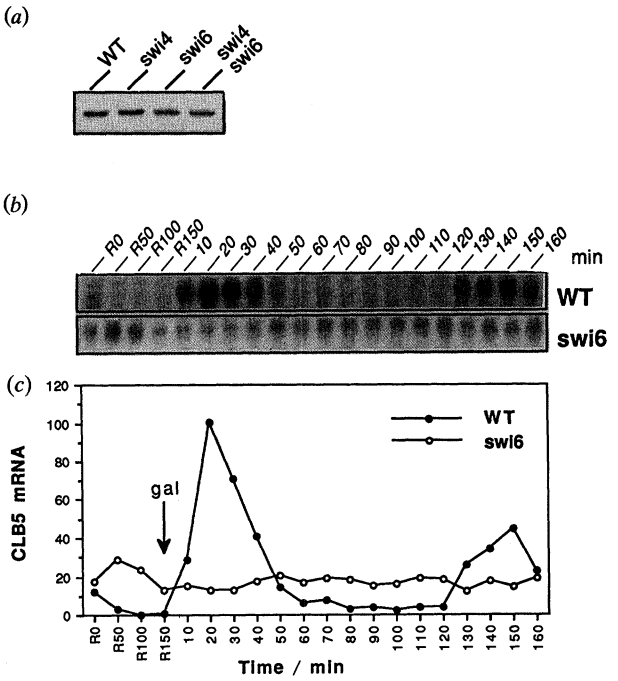

Figure 6. SW16 is required for the regulation of *CLB5* transcription. (*a*) Steady-state CLB5 transcript is not affected in *swi4* or *swi6* mutant strains. Northern analysis from exponentially growing wild-type (K2832), *swi4*Δ (K2833), *swi6*Δ (K2831) and *swi4*Δ, *swi6*Δ (K2392) all containing a rescuing *SpADH-CLN2* centromeric plasmid. A radio-labelled 0.5 kb internal EcoRI fragment of *CLB5* was used as a probe. (*b*) SWI6-dependent regulation of *CLB5* mRNA during the cell cycle. Northern analysis of RNA from congenic strains K2771 (WT) and K2786 (*swi6*) arrested for 2.5 h in YEP-raffinose (R150) by CLN depletion and released synchronously by addition of 2% galactose at $t=0$ min (Dirick *et al.* 1992). (*c*) Quantification of the above using a PhosphoImager (Molecular Dynamics); closed circles, K2771 (WT); open circles, K2786 (*swi6::TRP1*).

regulated, that is, they are absent in early G1, appear abruptly as cells undergo Start, and dissappear soon afterwards (figure 6*b*). The promoter region of *CLB5* has several sequences that are a good match to the MCB consensus. Two pieces of evidence suggest that *CLB5* might be regulated by MBF. First, a factor that contains Swi6 but not Swi4 binds to a promoter fragment from the *CLB5* promoter (figure 7). Second, the regulation of *CLB5* transcripts is abolished in *swi6* mutants, where it seems to be expressed constitutively throughout the cell cycle (figure 6*b*).

3. CONCLUSIONS

This article began with the question of what happens inside a yeast cell when it undergoes Start. Until recently, the prevailing view was that transcriptional controls would not be an important aspect. One reason for this view was that it was known that cleavage embryos can go through many cell cycles without any transcription. The second reason was the observation that most yeast cell cycle gene products are present in excess and do not need to be re-

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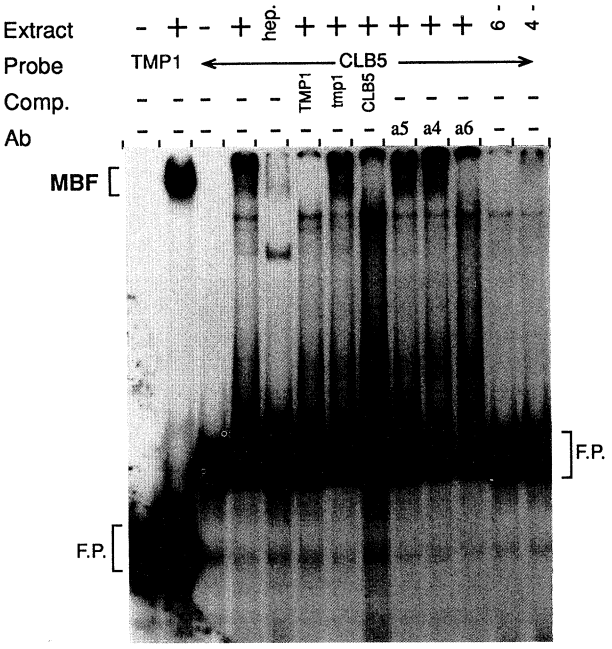

Figure 7. MBF binds to MCBs within the *CLB5* promoter. Gel retardation assays were performed using whole-cell extracts from wild-type (+), *swi6* mutant (K1354, 6-), *swi4* mutant (K1071, 4-) strains or p120-enriched heparin fraction (hep.) and a radiolabelled 110-b.p. PCR fragment containing four MCB motifs from the *CLB5* promoter (CLB5); TMP1, MCB oligonucleotide from the *TMP1* promoter (Dirick *et al.* 1992). MBF complex formation was specifically competed with a 50-fold molar excess of cold *TMP1* or *CLB5* probes but not by a mutant *TMP1* oligonucleotide where both MluI sites contain a point mutation (tmp1; Dirick *et al.* 1992). The MBF complex formed on the CLB5-MCB is supershifted by anti-Swi6 (a6) but not by anti-Swi5 (a5) or anti-Swi4 (a4) antibodies (1:20 dilution of sera). F.P., free probe.

synthesized each cell cycle for further cell cycle progression (Byers & Sowder 1980). It is now clear that cell cycle specific gene activation is vital for the entry into and progression through the mitotic programme of a yeast cell. G1 and G2-specific cyclins must be transcribed at Start and in G2 respectively.

The two types of promoter element known to confer late G1-specific transcription are bound by different but related transcription factors. SCB elements, which are found in the *HO*, *CLN2* and *HCS26* promoters, are bound by a factor (SBF) composed of the Swi4 and Swi6 proteins. MCB elements, which are found in the promoters of most genes involved in DNA replication, are bound by a factor composed of a 115 kDa protein (p120) and Swi6 (figure 8). Mutations in the *SWI4* gene have shown that SBF has an important role in the activation of the G1 cyclin genes *CLN1* and *CLN2*. It has so far not been possible to evaluate the role of MBF because the gene for p120 has not been identified. Our purification of MBF has allowed us to isolate sufficient quantities of p120 that it will be possible to derive peptide sequences useful for identifying its gene. One reason for believing that MBF (like SBF) will prove important for cell cycle progression is the discovery that it probably regulates *CLB5*, which encodes a new B-type cyclin expressed in late G1 and implicated in S phase progression (Epstein & Cross 1992; Schwob & Nasmyth 1993).

An obvious question is whether the transcriptional programme regulated by SBF and MBF is a conserved feature of the eukaryotic cell cycle. The *cdc10* gene from the fission yeast *Schizosaccharomyces pombe* is required for Start (Nurse *et al.* 1976), encodes a protein homologous to Swi6 (Breeden & Nasmyth 1987*b*), and is part of an MBF or DSC1-like factor that binds to MBCs (Lowndes *et al.* 1992*a*). It thus

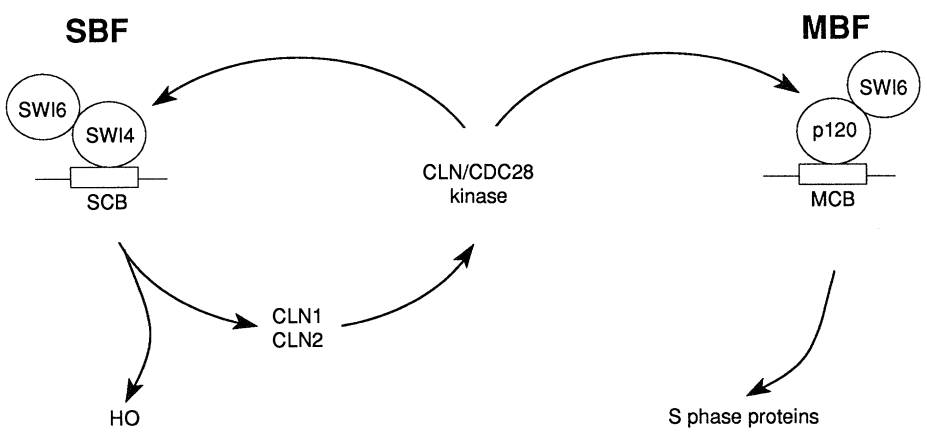

Figure 8. Transcriptional controls at Start. Two classes of genes are activated at Start. The first includes *HO*, *CLN1*, *CLN2*, and *HCS26*, which are activated via the binding of the SBF transcription factor to SCB elements (Andrews & Herskowitz 1989*b*; Nasmyth & Dirick 1991; Ogas *et al.* 1991). The second includes most genes involved in DNA replication, which are probably activated via the binding of the MBF or DSC1 transcription factor to MCB elements (Lowndes *et al.* 1991; Dirick *et al.* 1992). SBF and MBF are related factors whose regulation is similar and which share a common regulatory subunit, Swi6 (Taba *et al.* 1991; Lowndes *et al.* 1992*b*; Dirick *et al.* 1992*b*). The activation of G1 cyclins by SBF occurs via a positive feedback loop and is an important feature of Start (Dirick & Nasmyth 1991).

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seems that the SBF and MBF transcription factors may be conserved amongst fungi. G1 cyclins have also been discovered in mammalian cells (Koff *et al.* 1991). Cyclin E transcripts appear in late G1 at around the time of the restriction point but nothing is yet known about the transcription factor responsible for this regulation. So far, no protein homologous to Swi4 or Swi6 has been discovered in mammalian cells.

REFERENCES

Amon, A., Surana, U., Muroff, I. & Nasmyth, K. 1992 Regulation of $p34^{CDC28}$ tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. *Nature*, *Lond.* **355**, 368–371.

Andrews, B.J. & Herskowitz, I. 1989*a* The yeast SWI4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription. *Nature*, *Lond.* **342**, 830–833.

Andrews, B.J. & Herskowitz, I. 1989*b* Identification of a DNA binding factor involved in cell-cycle control of the yeast HO gene. *Cell* **57**, 21–29.

Andrews, B.J. & Herskowitz, I. 1990 Regulation of cell-cycle dependent gene expression in yeast. *J. biol. Chem.* **265**, 14057–14060.

Barberis, A., Widenhorn, K., Vitelli, K. & Busslinger, M. 1990 A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. *Genes Dev.* **4**, 849–859.

Breeden, L. & Nasmyth, K. 1987*a* Cell cycle control of the yeast HO gene: cis- and trans-acting regulators. *Cell* **48**, 389–397.

Breeden, L. & Nasmyth, K. 1987*b* Similarity between cell-cycle genes of budding yeast and fission yeast and the *Notch* gene of *Drosophila*. *Nature*, *Lond.* **329**, 651–654.

Byers, B. & Sowder, L. 1980 Gene expression in the yeast cell cycle. *J. Cell Biol.* **87**, 6a.

Byers, B. 1981 The Cytology of the yeast life cycle. In *The molecular biology of the yeast Saccharomyces: life cycle and inheritance* (ed. J. N. Strathern, E. W. Jones & J. R. Broach), pp. 59–96. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Carter, B.L.A. & Sudbery, P.E. 1980 Small-sized mutants of *Saccharomyces cerevisiae*. *Genetics* **96**, 561–566.

Cross, F. & Tinkelenberg, A.H. 1991 A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell* **65**, 875–883.

Dirick, L. & Nasmyth, K. 1991 Positive feedback in the activation of G1 cyclins in yeast. *Nature*, *Lond.* **351**, 754–757.

Dirick, L., Moll, T., Auer, H. & Nasmyth, K. 1992 A central role for SWI6 in modulating cell cycle *START* specific transcription in yeast. *Nature*, *Lond.* **357**, 508–513.

Epstein, C.B. & Cross, F.R. 1992 CLB5: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev.* **6**, 1695–1706.

Evans, T., Rosenthal, E.T., Youngbloom, J., Distel, D. & Hunt, T. 1983 Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**, 389–396.

Fitch, I., Dahman, C., Surana, U., Amon, A., Nasmyth, K., Goetsch, L., Byers, B. & Futcher, B. 1992 Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae*. *Molec. Biol. Cell.* **3**, 805–818.

Gordon, C.B. & Campbell, J.L. 1991 A cell cycle-responsive transcriptional control element and a negative control element in the gene encoding DNA polymerase alpha in *S. cerevisiae*. *Proc. natn. Acad. Sci. U.S.A.* **88**, 6058–6062.

Haid, A. & Suissa, M. 1983 Immunochemical identification of membrane proteins after sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Meth. Enzymol.* **96**, 192–205.

Herskowitz, I. 1989 A regulatory hierarchy for cell specialization in yeast. *Nature*, *Lond.* **342**, 749–757.

Kadonaga, J.T. & Tjian, R. 1986 Affinity purification of sequence-specific DNA binding proteins. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5889–5893.

Kim, H.B., Haarer, B.K. & Pringle, J.R. 1991 Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the CDC3 gene product and the timing of events at the budding site. *J. Cell Biol.* **112**, 535–544.

Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellec, K., Philippe, M. & Roberts, J.M. 1991 Human cyclin E, a new cyclin that interacts with two embers of the CDC2 gene family. *Cell* **66**, 1217–1228.

Laemmli, U.K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *Lond.* **227**, 680–685.

Lorincz, A.T. & Reed, S.I. 1984 Primary structure homology between the product of yeast division control gene CDC28 and vertebrate oncogenes. *Nature*, *Lond.* **307**, 183–185.

Lowndes, N.F., Johnson, A.L. & Johnston, L.H. 1991 Coordination of expression of DNA synthesis genes in budding yeast by a cell-cycle regulated trans factor. *Nature*, *Lond.* **350**, 247–250.

Lowndes, N.F., McInerny, C.J., Johnson, A.L., Fantes, P.A. & Johnston, L.H. 1992*a* Control of DNA synthesis genes in fission yeast by the cell-cycle gene cdc10+. *Nature*, *Lond.* **355**, 449–453.

Lowndes, N.F., Johnson, A.L., Breeden, L. & Johnston, L.H. 1992*b* SWI6 protein is required for transcription of the periodically expressed DNA synthesis genes in budding yease. *Nature*, *Lond.* **357**, 505–508.

McIntosh, E., Atkinson, T., Storms, R. & Smith, M. 1991 Characterization of a short, cis-acting DNA sequence which conveys cell cycle stage-dependent transcription in *Saccharomyces cerevisiae*. *Molec. cell. Biol.* **11**, 329–337.

Nash, R., Tokiwa, G., Anand, S., Erickson, K. & Futcher, A.B. 1988 The WH11+ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. *EMBO J.* **7**, 4335–4346.

Nasmyth, K.A. 1983 Molecular analysis of a cell lineage. *Nature*, *Lond.* **302**, 670–676.

Nasmyth, K. 1985 A repetitive DNA sequence that confers cell-cycle START (CDC28)-dependent transcription of the HO gene in yeast. *Cell* **42**, 225–235.

Nasmyth, K. & Shore, D. 1987 Transcriptional regulation in the yeast cell cycle. *Science*, *Wash.* **237**, 1162–1170.

Nasmyth, K. & Dirick, L. 1991 The role of *SWI4* and *SWI6* in the activity of G1 cyclins in yeast. *Cell* **66**, 995–1013.

Nurse, P., Thuriaux, P. & Nasmyth, K. 1976 Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. *Molec. gen. Genet.* **146**, 167–178.

Ogas, J., Andrews, B.J. & Herskowitz, I. 1991 Transcriptional Activation of CLN1, CLN2, and a Putative New G1 Cyclin (HCS26) by SWI4, a Positive Regulator of G1-Specific Transcription. *Cell* **66**, 1015–1026.

Pardee, A.B. 1989 G1 events and regulation of cell proliferation. *Science*, *Wash.* **246**, 603–608.

Pines, J. 1992 Cell proliferation and control. *Curr. Op. Cell Biol.* **4**, 144–148.

Pizzagalli, A., Valsasnini, P., Plevani, P. & Lucchini, G.

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1988 DNA polymerase I gene of *Saccharomyces cerevisiae*: Nucleotide sequence, mapping of a temperature-sensitive mutation, and protein homology with other DNA polymerases. *Proc. natn. Acad. Sci. U.S.A.* **85**, 772–3776.

Price, C., Nasmyth, K. & Schuster, T. 1991 A general approach to the isolation of cell cycle-regulated genes in the budding yeast, *Saccharomyces cerevisiae*. *J. molec. Biol.* **218**, 543–556.

Primig, M., Sockanathan, S., Auer, H. & Nasmyth, K. 1992 Anatomy of a transcription factor important for the start of the cell cycle in *Saccharomyces cerevisiae*. *Nature, Lond.* **358**, 593–597.

Pringle, J.R. & Hartwell, L.H. 1981 The *Saccharomyces cerevisiae* cell cycle. In *The molecular biology of the yeast Saccharomyces: life cycle and inheritance* (ed. J. N. Strathern, E. W. Jones & J. R. Broach), pp. 97–142. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Reed, S.I. 1980 The selection of *S. cerevisiae* mutants defective in the start event of cell division. *Genetics* **95**, 561–577.

Reid, B. & Hartwell, L.H. 1977 Regulation of mating in the cell cycle of *S. cerevisiae*. *J. Cell Biol.* **75**, 355–365.

Richardson, H.E., Wittenberg, C., Cross, R. & Reed, S.I. 1989 An essential G1 function for cyclin-like proteins in yeast. *Cell* **59**, 1127–1133.

Schwob, E. & Nasmyth, K. 1993 CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *S. cerevisiae*. *Genes Dev.* (In the press.)

Shore, D. & Nasmyth, K. 1987 Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. *Cell* **51**, 721–732.

Solomon, M.J., Glotzer, M., Lee, T.H., Philippe, M. & Kirschner, M.W. 1990 Cyclin activation of p34cdc2. *Cell* **63**, 1013–1024.

Sorger, P.K., Ammerer, G. & Shore, D. 1989 Identification and purification of sequence-specific DNA-binding proteins. In *Protein function: a practical approach* (ed. T. E. Creighton), pp. 199–278. Oxford: IRL Press.

Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A.B. & Nasmyth, K. 1991 The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* **65**, 145–161.

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B. & Nasmyth, K. 1993 Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.* **12**. (In the press.)

Taba, M.R., Muroff, I., Lydall, D., Tebb, G. & Nasmyth, K. 1991 Changes in a SWI4,6-DNA-binding complex occur at the time of *HO* gene activation in yeast. *Genes Dev.* **5**, 2000–2013.

Tyers, M., Tokiwa, G. & Futcher, B. 1993 Comparison of the *S. cerevisiae* G1 cycins: Cln3 may be an upstream activator of Cln1, Cln2, and other cyclins. *EMBO J.* (In the press.)

Wittenberg, C., Sugimoto, K. & Reed, S.I. 1990 G1-specific cyclins of *S. cerevisiae*: Cell cycle periodicity, Regulation by mating pheromone, and association with the p34CDC28 protein kinase. *Cell* **62**, 225–237.

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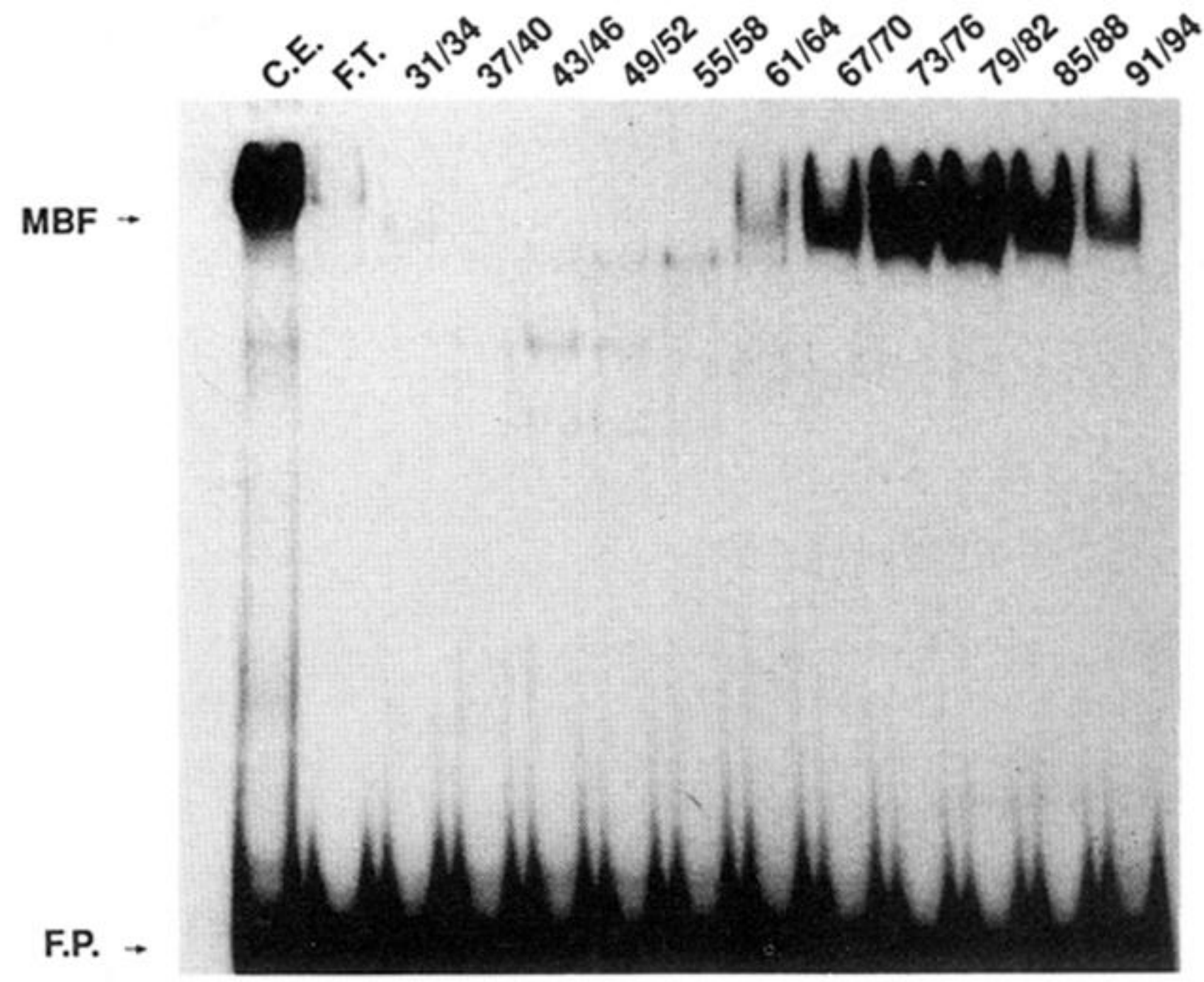

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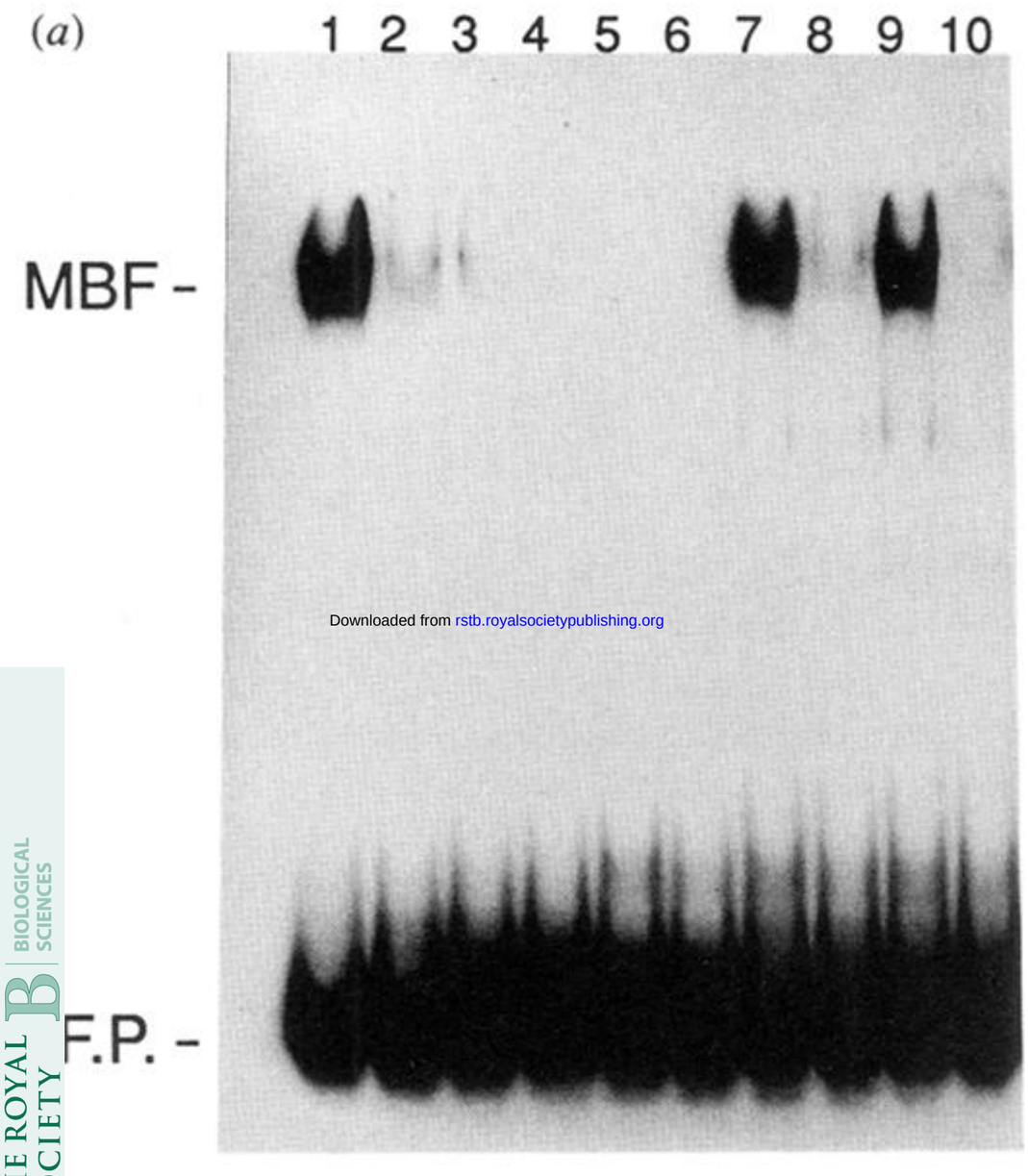
gure 2. Heparin fractionation of yeast extracts. Yeast
ıole-cell extracts were prepared and fractionated over a
parin agarose column as described in Materials and
ethods. Gel retardation assays were performed on crude
tracts (c.c., 2 µl), the flow through (f.t., 2 µl), as well as
ctions eluted from the column using a 50–600 mM
$H_4)_2SO_4$ gradient. Aliquots from two nearby fractions
µl) were pooled as indicated and mixed with 0.5 ng of
gonucleotide (corresponding to 10^4 c.p.m.) for each DNA
ıding assay. MBF (top panel) and SBF (bottom panel)
re assayed using probes derived from the *TMP1* and the
N2 promoter respectively. The volumes of crude extract
5 mg ml^{-1} protein) and of fractions containing high levels
MBF (0.2 mg ml^{-1} protein) were both approximately
0 ml. We therefore estimate a 40–50% recovery with a
–60-fold purification. The free probe is labelled with F.P.



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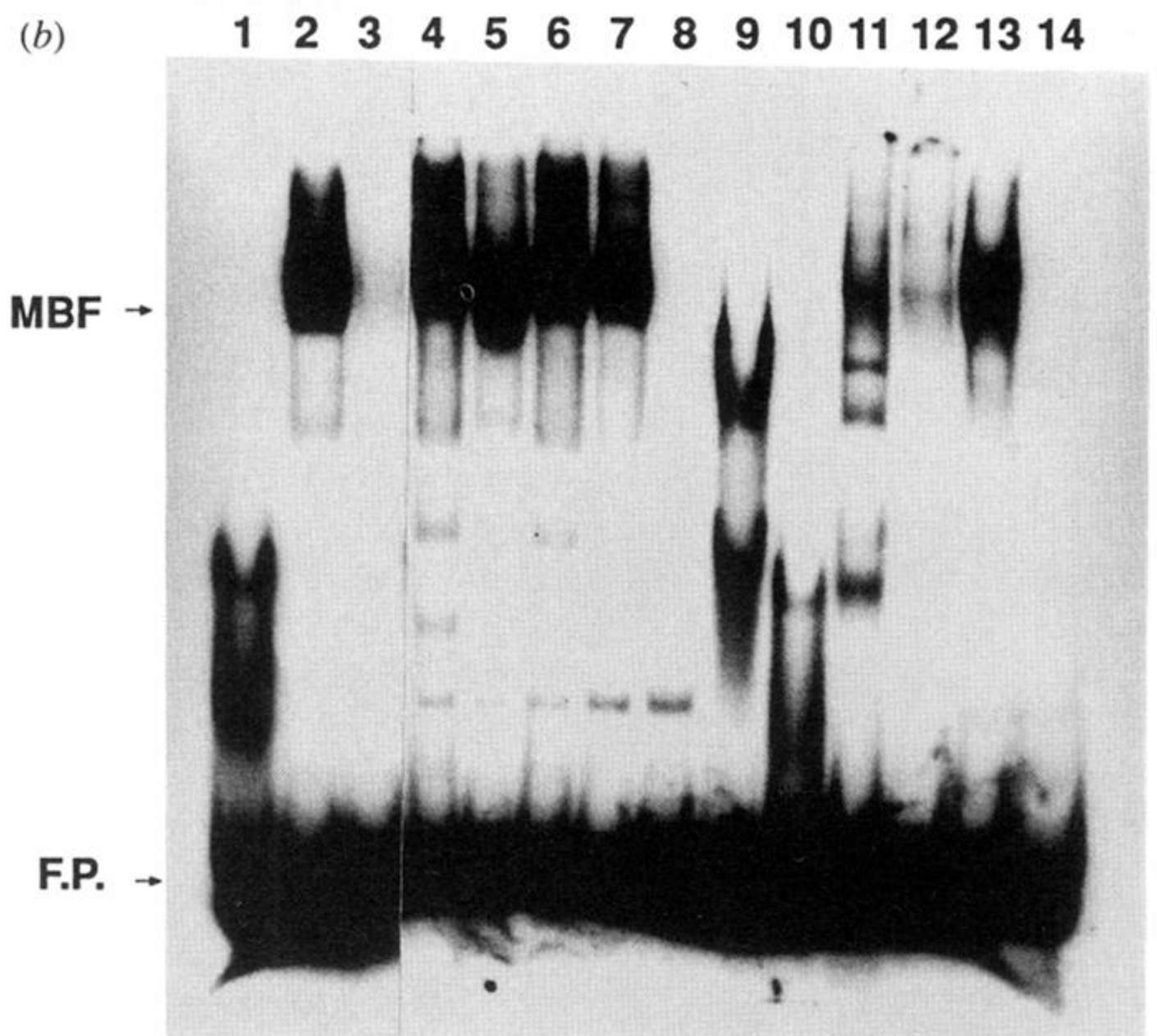

gure 3. (*a*) MBF activity can be reconstituted after DNA affinity chromatography. Both untreated heparin arose eluates containing MBF activity (lanes 1, 3 and 5) as well as eluates pre-incubated with anti-Swi6 antibodies upled to sepharose beads (lanes 2, 4 and 6) were fractionated over a specific MCB-containing DNA-affinity lumn. Gel retardation assays were performed on 2 µl of both untreated (lane 1) and anti-Swi6 treated (lane 2) parin agarose eluates, the respective flow throughs (lanes 3 and 4) and the DNA column eluates (lanes 5 and 6). BF activity is reconstituted upon mixing untreated DNA column eluates with either untreated or anti-Swi6 ated flow through (lanes 7 and 9, 2 µl each) during the DNA binding reaction, but not when mixing immuno-pleted DNA column eluates with the respective flow throughs (lanes 8 and 10, 2 µl each). More than 70% of the BF activity could be recovered in the eluates after DNA chromatography, as estimated from the mixing periments. DNA chromatography therefore gives a 200–300-fold purification of MBF. The free probe is labelled th F.P. (*b*) Material eluted from the DNA affinity column was assayed for MBF activity in the presence of various ude protein preparations. Lane 1, eluate only; lane 2, eluate preincubated with flow through of DNA column; lane flow through assayed in the absence of eluate; lane 4, eluate preincubated with 50 µg reticulocyte lysate; lane 5, me as lane 4 but reticulocyte lysate was heated to 75°C for 8 min Before preincubation with eluate; lanes 6 and 7, in lane 4; lane 8, reticulocyte lysate assayed in the absence of column eluate; lane 9, eluate preincubated with µg BSA; lane 10, eluate preincubated with 20 µg insulin; lane 11, preincubation with 20 µg *E. coli* extract; lane , as in lane 11 but after boiling the *E. coli* extract for 15 min; lane 13, 40 µg crude yeast extract was heated to 90°C 10 min and then preincubated with column eluate; lane 14, heat treated yeast extract assayed in the absence of uate.

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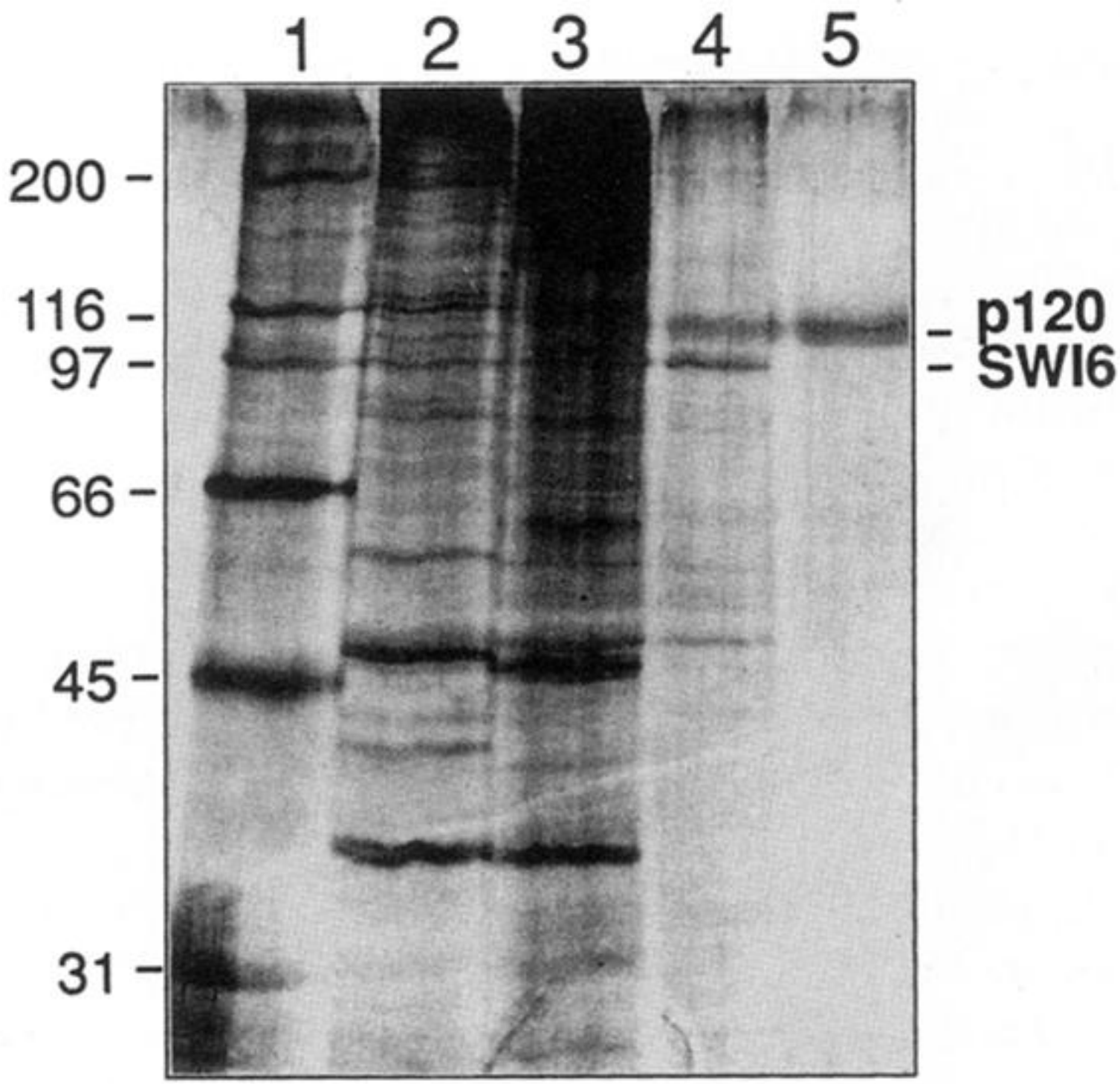

gure 4. Purification of p120. Proteins present in whole cell tracts (lane 2), after heparin agarose chromatography ne 3), DNA affinity chromatography (lane 4) and gel rification (lane 5) were visualized by silver staining. p120 d Swi6 are outlined to the right. Molecular mass markers kDa are shown to the left (lane 1).

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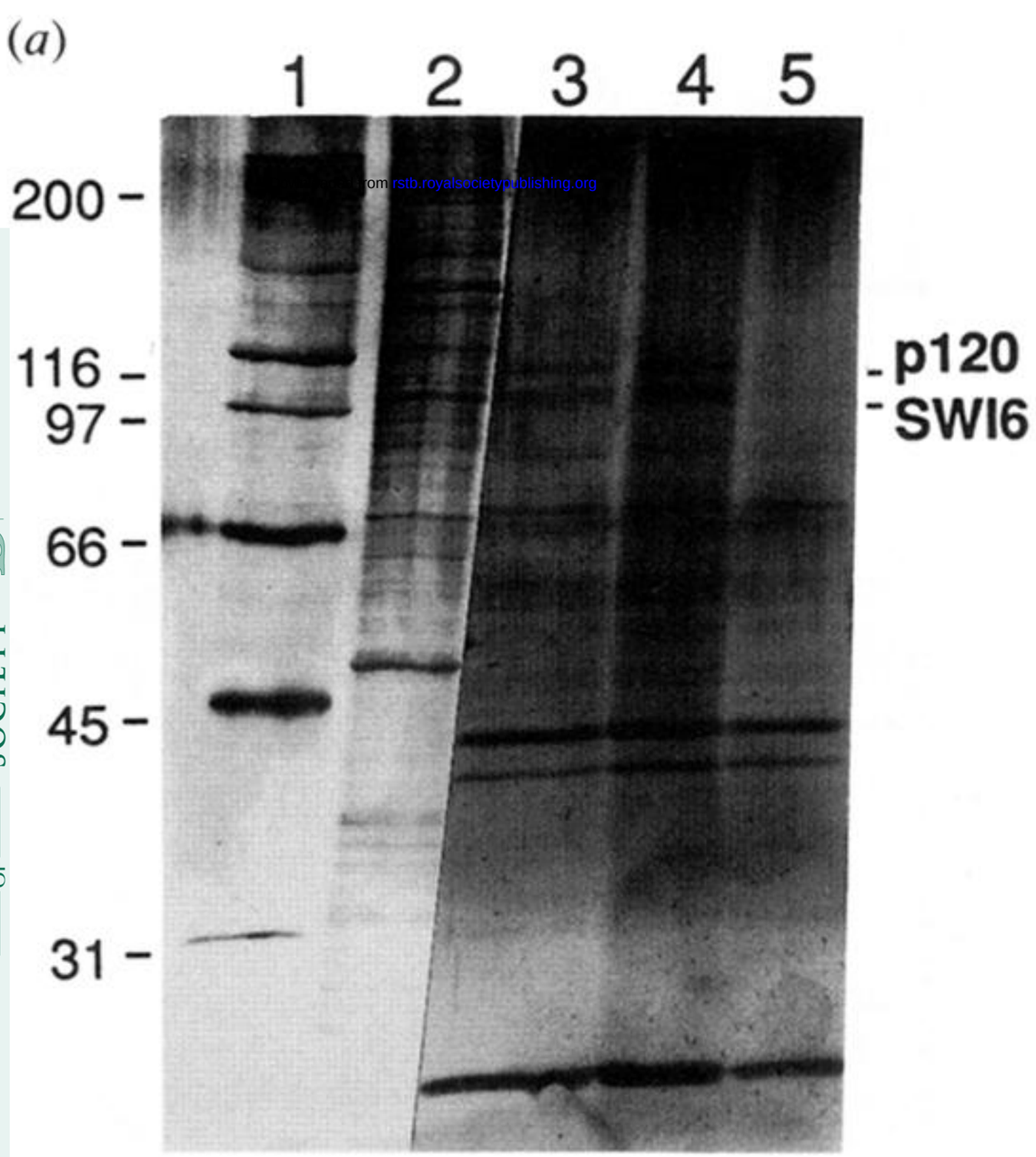

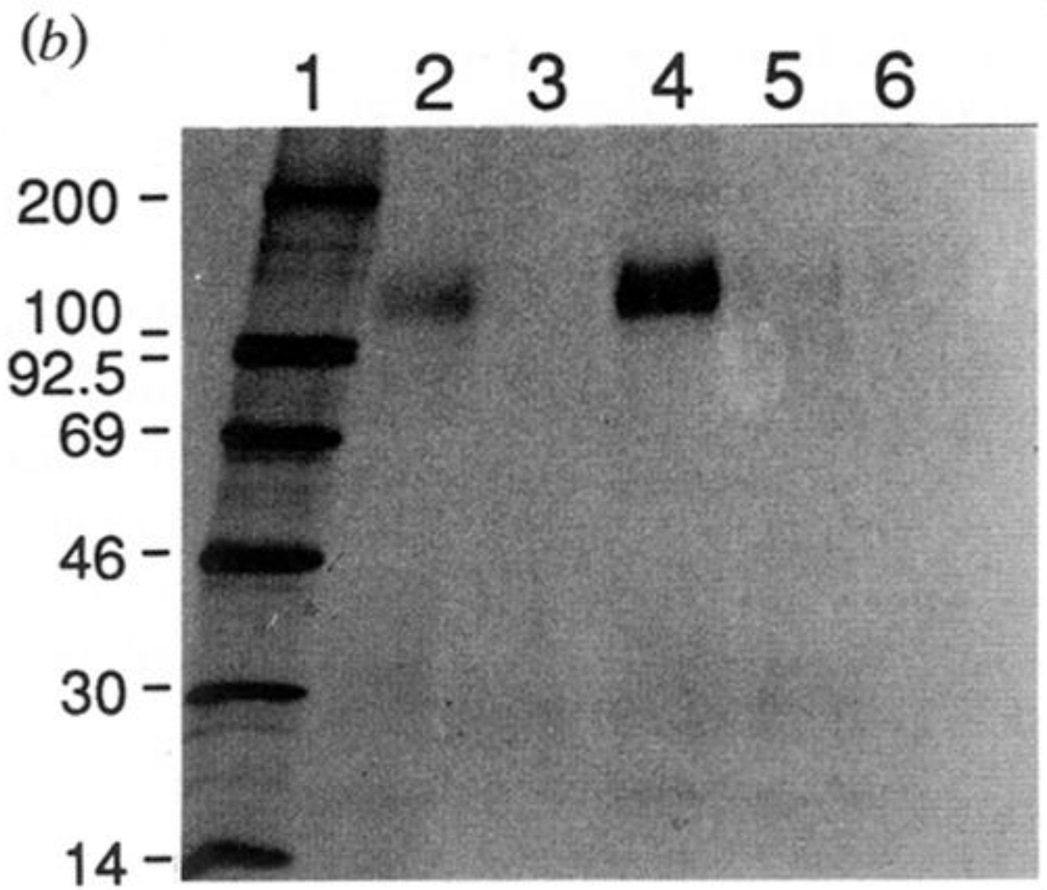

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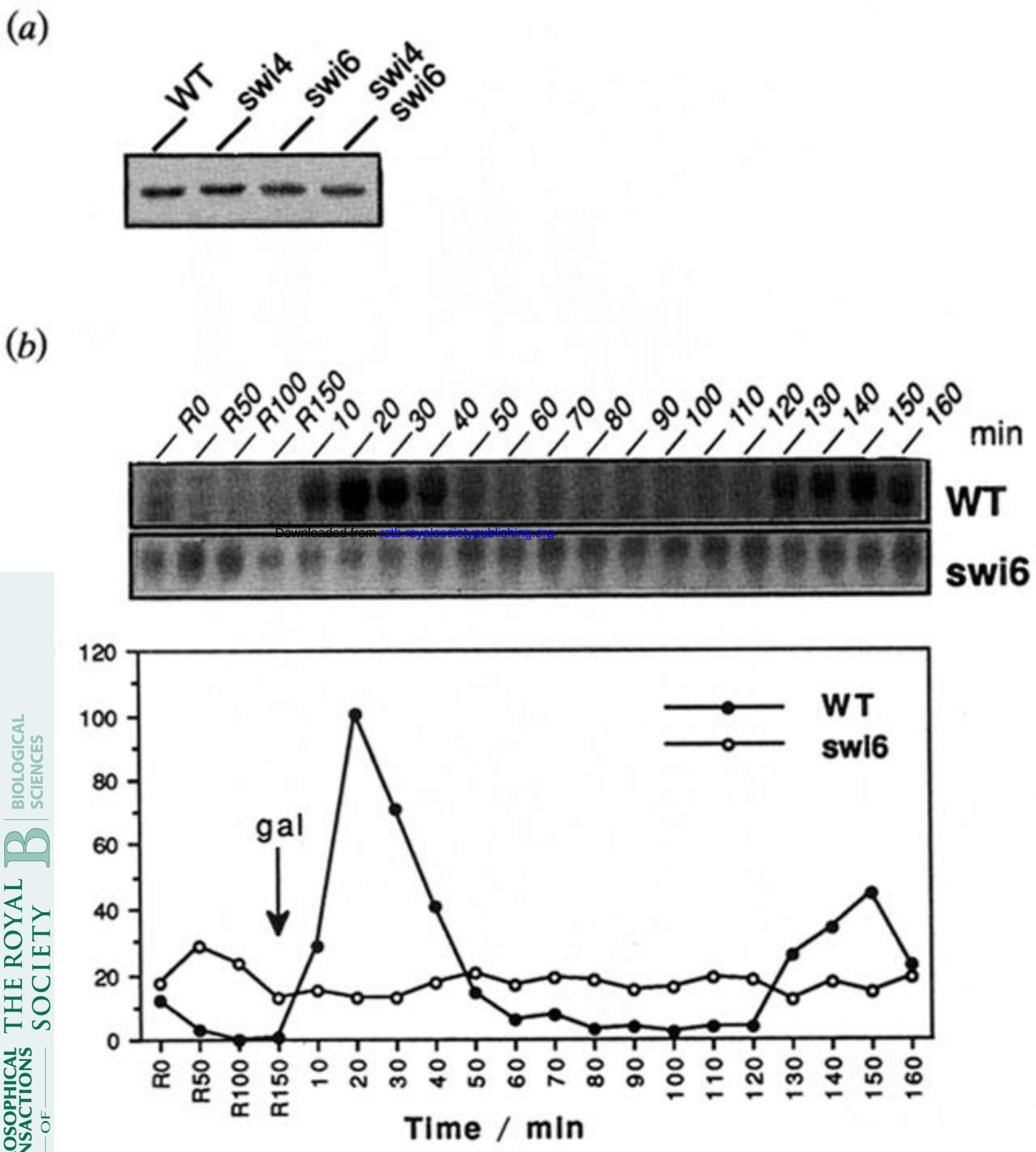
gure 5. p120 is retained on a specific DNA affinity column. (*a*) Silver staining of proteins. Lane 1: molecular mass arkers (in kDa). Lane 2: eluates from heparin agarose column containing the peak of MBF activity. Lanes 3 and 4: iates from DNA affinity column (eluates from two separate column runs are shown). Lane 5: eluates from DNA inity column after immunodepletion of heparin agarose eluates with Swi6-specific antibodies. (*b*) Detection of 20 by DNA-crosslinking and co-immunoprecipitation. After incubating radiolabelled MCB containing oligonuc-otides (a bromodexyuridine-substituted MCB-TMP1 oligonucleotide; see Dirick *et al.* (1992) for experimental tails) with heparin agarose eluates containing MBF activity, protein–DNA complexes were covalently crosslinked uv-irradiation. Cross-linked complexes were immunoprecipitated with Swi6-specific (lanes 2–4), Swi4-specific ne 5) or DHFR-specific (lane 6) antibodies after digestion with DNaseI. Either no competitor (lanes 2, 5, and 6), 50-fold molar excess unlabelled MCB-TMP1 (lane 3), or a 50-fold molar excess of unlabelled point-mutated CB-TMP1 (both of whose MCBs are mutated, lane 4) were added during the binding reactions.



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gure 6. SW16 is required for the regulation of *CLB5* nscription. (*a*) Steady-state CLB5 transcript is not ected in *swi4* or *swi6* mutant strains. Northern analysis m exponentially growing wild-type (K2832), *swi4*Δ 2833), *swi6*Δ (K2831) and *swi4*Δ, *swi6*Δ (K2392) all ntaining a rescuing *SpADH-CLN2* centromeric plasmid. A dio-labelled 0.5 kb internal EcoRI fragment of *CLB5* was ed as a probe. (*b*) SWI6-dependent regulation of *CLB5* RNA during the cell cycle. Northern analysis of RNA m congenic strains K2771 (WT) and K2786 (*swi6*) rested for 2.5 h in YEP-raffinose (R150) by CLN depleand released synchronously by addition of 2% galactose $t=0$ min (Dirick *et al.* 1992). (*c*) Quantification of the ove using a PhosphoImager (Molecular Dynamics); sed circles, K2771 (WT); open circles, K2786 *vi6::TRP1*).

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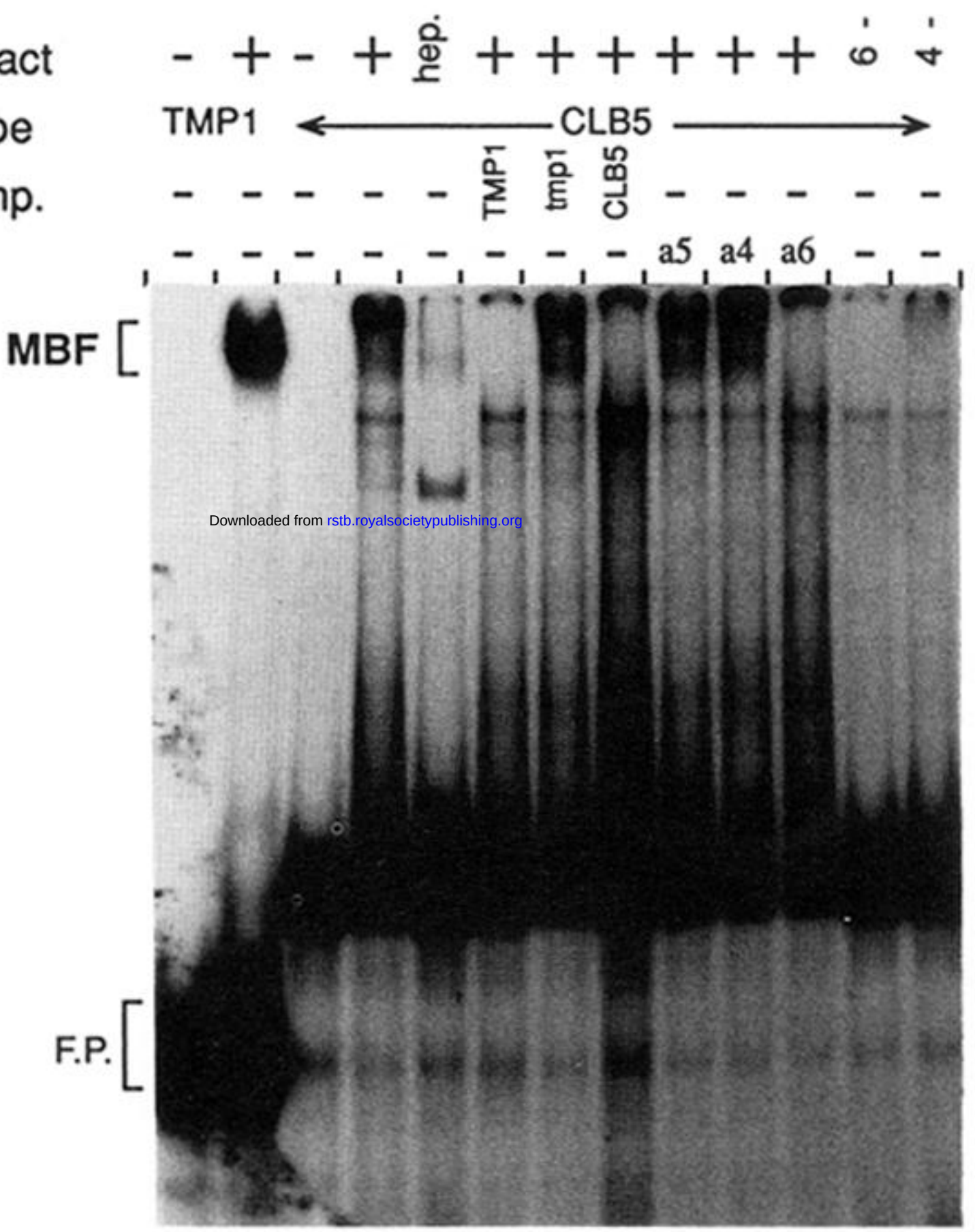

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gure 7. MBF binds to MCBs within the *CLB5* promoter. el retardation assays were performed using whole-cell tracts from wild-type (+), *swi6* mutant (K1354, 6-), *swi4* utant (K1071, 4-) strains or p120-enriched heparin frac- on (hep.) and a radiolabelled 110-b.p. PCR fragment ntaining four MCB motifs from the *CLB5* promoter LB5); TMP1, MCB oligonucleotide from the *TMP1* omoter (Dirick *et al.* 1992). MBF complex formation was ecifically competed with a 50-fold molar excess of cold *MP1* or *CLB5* probes but not by a mutant *TMP1* igonucleotide where both MluI sites contain a point utation (tmp1; Dirick *et al.* 1992). The MBF complex rmed on the CLB5-MCB is supershifted by anti-Swi6 (a6) ut not by anti-Swi5 (a5) or anti-Swi4 (a4) antibodies (1:20 lution of sera). F.P., free probe.

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