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Fine-Structure Analysis of Ribosomal Protein Gene Transcription

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The ribosomal protein genes of *Saccharomyces cerevisiae*, responsible for nearly 40% of the polymerase II transcription initiation events, are characterized by the constitutive tight binding of the transcription factor Rap1. Rap1 binds at many places in the yeast genome, including glycolytic enzyme genes, the silent MAT loci, and telomeres, its specificity arising from specific cofactors recruited at the appropriate genes. At the ribosomal protein genes two such cofactors have recently been identified as Fhl1 and Ifh1. We have now characterized the interaction of these factors at a bidirectional ribosomal protein promoter by replacing the Rap1 sites with LexA operator sites. LexA-Gal4(AD) drives active transcription at this modified promoter, although not always at the correct initiation site. Tethering Rap1 to the promoter neither drives transcription nor recruits Fhl1 or Ifh1, showing that Rap1 function requires direct DNA binding. Tethering Fhl1 also fails to activate transcription, even though it does recruit Ifh1, suggesting that Fhl1 does more than simply provide a platform for Ifh1. Tethering Ifh1 to the promoter leads to low-level transcription, at the correct initiation sites. Remarkably, activation by tethered LexA-Gal4(AD) is strongly reduced when TOR kinase is inhibited by rapamycin. Thus, TOR can act independently of Fhl1/Ifh1 at ribosomal protein promoters. We also show that, in our strain background, the response of ribosomal protein promoters to TOR inhibition is independent of the Ifh1-related protein Crf1, indicating that the role of this corepressor is strain specific. Fine-structure chromatin mapping of several ribosomal protein promoters revealed that histones are essentially absent from the Rap1 sites, while Fhl1 and Ifh1 are coincident with each other but distinct from Rap1.

The 138 genes encoding the 79 ribosomal proteins (RP) of *Saccharomyces cerevisiae* are arguably the most coordinately regulated cluster of genes, spread throughout the yeast genome (7, 11). It was originally thought that the basis for much of this regulation lay in the presence of binding sites for the protein Rap1 upstream of a large majority of the RP genes (17, 21).

However, Rap1 is a protein of many functions (reviewed by references 28 and 29). It is the primary transcription factor for the glycolytic genes and several translation factor genes. It acts as the major duplex DNA binding protein of telomeres. It nucleates the silencing of the HML and HMR mating-type loci. Genome-wide chromatin immunoprecipitation (ChIP) analysis revealed that Rap1 binds to about 5% of yeast genes and participates in the activation of 37% of RNA polymerase II transcripts in exponentially growing yeast cells (21). There is good evidence that the initial step of Rap1 is to clear nucleosomes from a patch of DNA (28, 40) and that the second step is to recruit specific factors to carry out the appropriate function. It is now clear that for the RP genes these factors are Fhl1 and Ifh1, which are found almost exclusively at RP genes. Gcr1 and Gcr2 are present at many glycolytic enzyme genes. Sir3, Sir4, and others are recruited for silencing at the silent MAT loci and telomeres (19, 21, 34).

ChIP analysis of the RP genes showed that both Rap1 and Fhl1 are constitutively found at the promoters. Only occupancy by Ifh1 is correlated with active transcription, suggesting that Ifh1 plays a central role in the regulation of RP gene transcription (26, 33, 34, 39). Rap1 is one of the DNA binding proteins for which many consensus sequences have been suggested (29). Interestingly, the Rap1-binding sequences at RP gene promoters, termed RPG boxes, are quite different from those at the telomeres, while those at glycolytic gene promoters appear to be in between. Yet the basis for specificity remains obscure, although it has been suggested that Rap1 undergoes distinct conformational changes as a result of binding to somewhat different sequences (29).

At the RP genes, it has been proposed that Rap1 recruits not only TAFs, which in turn recruit TATA binding protein to the RP genes that have characteristically poor TATA boxes (27), but also Esa1, which could acetylate either histone H4 or another participant in transcriptional activation (31). Yet, Esa1 probably provides little specificity since by ChIP analysis it is found upstream of many actively transcribed genes (30, 32).

While genome-wide ChIP analysis revealed that Rap1, Fhl1, and Ifh1 are recruited to a majority of the RP gene promoters (19, 34, 39), neither the basis for the recruitment nor the role played by the factors in transcription of RP genes was clear. Furthermore, the binding sites for Fhl1 and for Ifh1 are elusive. When assayed in vitro, neither Fhl1 nor Ifh1 binds RP promoters, either by itself or in the presence of Rap1 (33). Ifh1 appears to be recruited to RP promoters through its interaction with the "forkhead-associated" (FHA) domain of Fhl1 (9, 26, 33, 34). However, the story must be more complex. Although the FHA domain of Fhl1 can recruit Ifh1 to serve as a

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TABLE 1. Strains used in this work[a]

Strain	Genotype	Reference or source
W303**a** and α	*ade2-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 can1-100 ssd1-1*	36
DR70	W303α *IFH1-Myc13*::*HIS3MX6 FHL1-HA3*::*G418r*	This study
YZ160	W303**a** *URA3*::pRS306-ConI	This study
YZ170	DR70 *URA3*::pRS306-ConI	This study
YZ171	DR70 *URA3*::pRS306-ConI-BN	This study
YZ172	DR70 *URA3*::pRS306-ConI-BN-LexAx8	This study
YZ162	W303**a** *URA3*::pRS306-ConI-BN-LexAx8	This study
DR34	W303α *FHL1*Δ::*HIS3*	33
YZ147	W303α *His3MX6*:*PGAL1-3HA-IFH1*	33
SCR101	W303-1b P(USA_{GAL}-rpL25 hybrid promoter)-*RAP1*	13
TB50**a**	*MAT***a** *leu2-3,112 ura3-52 trp1 his3 rme1 HMLa*	26
DM45-2C	TB50**a** *crf1*Δ::*KanMX6*	26
YSS120	W303α *crf1*Δ::*URA3*	This study

[a] pRS306-ConI contains the *RPL24A*/*RPL30* promoter driving $G418^r$ and GFP, respectively (43). pRS306-ConI-BN contains the *RPL24A*/*RPL30* promoter with Rap1 sites deleted. pRS306-ConI-BN-LexAx8 contains the *RPL24A*/*RPL30* promoter with its Rap1 sites replaced with eight copies of LexA operators (Fig. 1).

transcriptional activator of a GAL-based artificial reporter, a nearly full length Fhl1 recruits nearly as much Ifh1, but very little transcription ensues (34). Indeed, Fhl1 has been proposed as a repressor of RP gene transcription (5, 14). Furthermore, there is no direct evidence that Ifh1 functions as a transcriptional activator in the context of an RP gene promoter.

Utilizing a minimally engineered promoter that drives the transcription of two RP genes oriented head-to-head, we have found that for Rap1 to recruit Fhl1 and Ifh1 and to activate transcription, it must bind DNA directly. Furthermore, Rap1 binding to sites from glycolytic genes, within the context of the RP genes, recruits neither Fhl1 nor Ifh1. Recruitment of Ifh1 by Fhl1 tethered to the promoter is also insufficient to drive transcription, although tethered Ifh1 alone does. By high-resolution ChIP analysis, we find that Fhl1 and Ifh1 are recruited to the RP promoters at a location distinct from the Rap1-binding sites.

MATERIALS AND METHODS

Strains and plasmid constructs. The strains used in this study are listed in Table 1. The epitope tagging of the proteins of interest was done by PCR-based gene targeting (23).

Plasmid pRS306-ConI contains the intergenic region of *RPL24A* and *RPL30*, which controls the expression of $G418^r$ and green fluorescent protein (GFP), respectively (Fig. 1) (43). The *RAP1* binding sites were replaced with two restriction sites (BglII and NheI) by PCR (pRS306-ConI-BN). To delete the Rap1-binding sites in the pRS306-ConI vector, primers (5′-CACTAAAATCTGAGATCAAAAATA TGTGagatctgctagcAAGGTCTTTTTCCAAGAAACGTATC-3′) and (5′-GATA CGTTTCTTGGAAAAAGACCTTgctagcagatctCACATATTTTTGATCTCAG ATTTTAGTG-3′ (lowercase letters are the sites for BglII and NheI) were used to generate PCR products using pRS306-ConI as template. The PCR product was incubated at 37°C with DpnI for 1 h, followed by ligation (in the presence of 1 μl polynucleotide kinase) and transformation to the XL1-Gold competent strain of *Escherichia coli*.

The *RAP1* sites were reinserted into the restriction sites by ligating an annealed oligonucleotide containing the original *RAP1* binding sites with pRS306-ConI-BN digested with BglII and NheI. Similarly, eight copies of LexA operators were amplified by PCR using pSH18-34 (Invitrogen) as the template and subcloned into the plasmid pRS306-ConI-BN.

Full-length open reading frames (ORFs) (starting at residue 2) of *RAP1*, *FHL1*, and *IFH1* were amplified by PCR using yeast genomic DNA as templates and subcloned in frame with the LexA DNA binding domain in the 2μm vector pBTM116 (1), generating pBTM116-RAP1, pBTM116-FHL1, and pBTM116-IFH1, respectively, which were verified by sequencing. The plasmid pLexA-pos contains LexA-Gal4(AD) under the control of the *ADH1* promoter (Clontech).

Northern and reverse transcription-PCR mRNA analysis. For Northern analysis, preparation of yeast total RNA and blotting was performed as described previously (43). The oligonucleotide JW61L was used to identify GFP and *RPL30* mRNAs, and JW2258 was used to identify $G418^r$ and *RPL24A* mRNAs (Table 2). For analysis by reverse transcription/real-time PCR, total RNA from 2×10^7 cells was isolated using the RNeasy minikit (QIAGEN). An 0.4-μg amount of DNase-treated total RNA was reverse transcribed. Quantitation of cDNAs was achieved using Taqman real-time PCR on an Applied Biosystems ABI Prism 7700 machine. Sequences of primer pairs and Taqman probes are available upon request.

ChIP. ChIP was carried out as described previously (16) with slight modifications. The antibodies used were anti-LexA rabbit polyclonal antibody (Santa Cruz), anti c-Myc mouse monoclonal antibody (9E10; Santa Cruz), antihemagglutinin (anti-HA) mouse monoclonal antibody (12CA5, hybridoma superna-

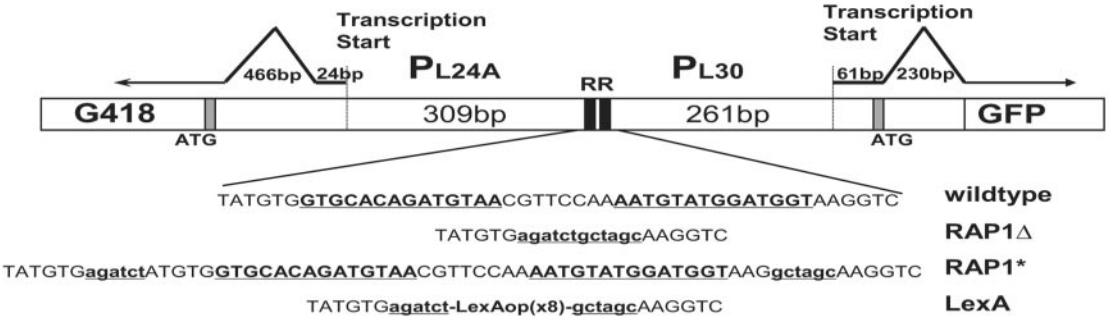

FIG. 1. Reporter constructs. The promoters of *RPL24A* and *RPL30*, which are divergently transcribed from an intergenic region of 618 bp, share two Rap1-binding sites, identified as "R," with the sequences underlined and in boldface. The ORFs of *RPL24A* and *RPL30* were replaced with those of $G418^r$ and GFP, respectively. The sequences of both transcription initiation sites and both introns remain intact. The distances from the Rap1-binding sites to the transcription start sites of *RPL24A* and *RPL30* are indicated. The underlined Rap1 sites were replaced by two restriction sites (BglII and NheI), which are in underlined, boldface lowercase (construct RAP1Δ). The Rap1-binding sites were reinserted at the restriction sites (RAP1*), with extra nucleotides on both sides. Alternatively, eight copies of the LexA operators (four tandem repeats of the sequence [GTATATAAAACCAGTGGTTATAT], which contains two LexA binding sites) were also inserted at the restriction sites (LexA). The reporter constructs were cloned in pRS306 (35) and, following cleavage with StuI, were inserted at the *URA3* locus by integrative transformation. Single-copy insertions were verified by PCR and Southern blotting.

TABLE 2. Oligonucleotide primers for Northern blotting and PCR

Primer	Sequence	Description
JW61L	CATCTCTGCGTATATTGATTAA	Oligonucleotide probe for *RPL30* and GFP
JW2258	CTCTCAAGTTATTGTCTTGGTGTC	Oligonucleotide probe for *RPL24A* and G418 resistance gene
JW2240	TCGAGGATGCGTTTTTAACC	Forward primer for ACT1 promoter region
JW2241	CAGGTTGACGTTCCCTTTGT	Reverse primer for ACT1 promoter region
JW2407	ATGTGGTGCACAGATGTAACG	Forward primer for L30-L24A promoter region
JW2408	AATTTAAATGCGGCCCTAGC	Reverse primer for L30-L24A promoter region
JW2411	GCGGGAAAGGGTTTAGTACC	Forward primer for PGK1 promoter region
JW2412	TGTCACACGATTCGGACAAT	Reverse primer for PGK1 promoter region
JW2429	TCACATCCACGTGACCAGTT	Forward primer for RPL11A promoter region
JW2430	AACTTTCGCATAGCTGAGTGG	Reverse primer for RPL11A promoter region
JW2231	TAATGCGGCTAGCAAGGTCT	Forward primer for CONI-BN-LEXA
JW2232	AATTTAAATGCGGCCCTAGC	Reverse primer for RPL30 promoter region
JW2259	TGTATGGATGGTAAGGCTAGCAA	Forward primer for ConI-BN-RAP1*

tant), anti-histone H3 C-terminal region antibody (ab1791; Abcam), and anti-Rap1 rabbit polyclonal antibody (34). Briefly, formaldehyde was added to 200 ml of culture (with an A_{600} of ~0.6 to 1.0) at a final concentration of 1% and incubated at room temperature for 20 min with occasional swirling and then 30 ml of 3 M glycine was added for 5 min. Chromatin was prepared as described previously (16). For the high-resolution ChIP (Fig. 8 and 9) the sonication time was extended, using 16 15-s bursts separated by 1 min of cooling on ice, so that the average size of sheared chromatin was ~200 bp. Extracts containing sheared chromatin were precleared with 20 μl UltraLink protein A/G beads (Pierce) for 3 hours at 4°C before being treated at 4°C overnight with specific antibody prebound to 20 μl protein A/G beads. Quantitative analyses were performed on an ABI PRISM 7900HT real-time PCR system (Applied Biosystems). To calculate the enrichment (*n*-fold) of LexA (or LexA fusion protein), Rap1, Fhl1-HA_3, and Ifh1-Myc_{13} occupancy at an individual promoter, the $2^{-\Delta\Delta CT}$ method (22) was applied, using a control promoter of *ACT1* that has been shown not to be occupied by Rap1, Fhl1p, or Ifh1p (21, 33). The oligonucleotide primers used in real-time PCR are listed in Table 2; those used for high-resolution analysis (Fig. 8 and 9) are available upon request.

RESULTS

A reporter construct to study ribosomal protein gene transcription. Because the function of Rap1 depends significantly on its context, analysis of the way in which it influences Fhl1 and Ifh1 requires a test gene that differs minimally from the wild-type (wt) sequence. Yet ribosome synthesis is essential to cell viability; manipulation of endogenous RP gene promoters or their transcription factors leads to detrimental effects on growth. To avoid these problems, we generated a reporter construct that maintains as intact as possible the structure of the RP gene promoter elements including the transcription start site (43). *RPL24A* and *RPL30* are divergently transcribed from an intergenic region of 609 bp that contains two Rap1-binding sites (Fig. 1) (6). The ORFs of *RPL24A* and *RPL30* were replaced with those of $G418^r$ and GFP, respectively. Since the sequences of the transcription start sites and introns of *RPL24A* and *RPL30* remained intact, the novel sequences are distant by 490 and 296 bp, respectively, from the sites of initiation of transcription. The construct was integrated as a single copy at the *URA3* locus. The reporter is regulated in the same way as an RP gene (20, 43; data not shown) and yet is dispensable to the cell.

The two Rap1-binding sites were replaced with two restriction sites by PCR mutagenesis (see Materials and Methods) (Fig. 1). Either the original Rap1-binding sites or eight copies of the LexA operator (10) were inserted. Thus, the reporter has maintained almost all *cis*-acting elements of an RP promoter except the known Rap1-binding sites.

LexA fusion proteins remain functional. Full-length ORFs of *RAP1*, *FHL1*, and *IFH1* were subcloned in frame with the LexA DNA binding domain in the 2μm vector pBTM116 (1) and, after verification of sequence fidelity, were introduced into strains carrying the test gene with the LexA binding sites described above (Fig. 1). Western blotting showed that the fusion proteins were expressed, although at substantially different levels (Fig. 2A). To determine whether the LexA fusion proteins were functional, we transformed the plasmids expressing LexA-Rap1 or LexA-Ifh1 into strains in which *RAP1* or

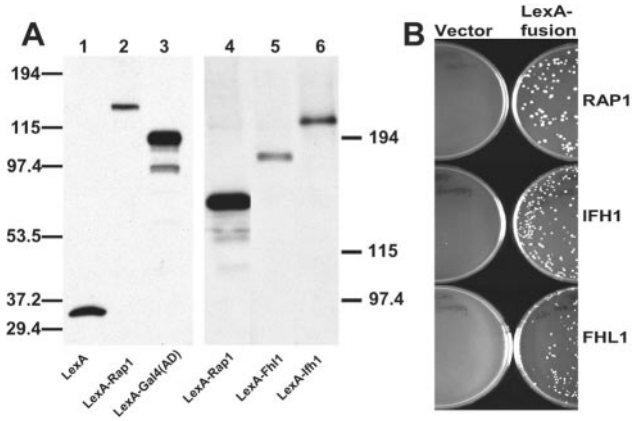

FIG. 2. LexA fusion proteins are expressed and remain fully functional. (A) The plasmids containing the LexA fusion proteins or the vector alone were transformed into the strain YZ172 (Table 1). Whole-cell extracts were prepared from log-phase cells and analyzed by Western blotting (left, 4 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis; right, 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The blot was probed with anti-LexA antibody followed by secondary antibody conjugated with horseradish peroxidase. Lane 1, LexA alone; lane 2, LexA-Rap1; lane 3, LexA-Gal4; lane 4, LexA-Rap1; lane 5, LexA-Fhl1; lane 6, LexA-Ifh1. Numbers at the sides of the panels show molecular masses in kilodaltons. (B) The plasmids containing the LexA fusion protein, or the vector alone, were transformed into a strain that has a growth defect on dextrose-containing medium and incubated for 3 days at 30°C. pBTM116-RAP1 was transformed into SCR101 (P_{GAL}-RAP1) (13). pBTM116-IFH1 was transformed into strain YZ147 (P_{GAL}-IFH1). pBTM116-FHL1 was transformed into strain DR34 (*fhl1*Δ).

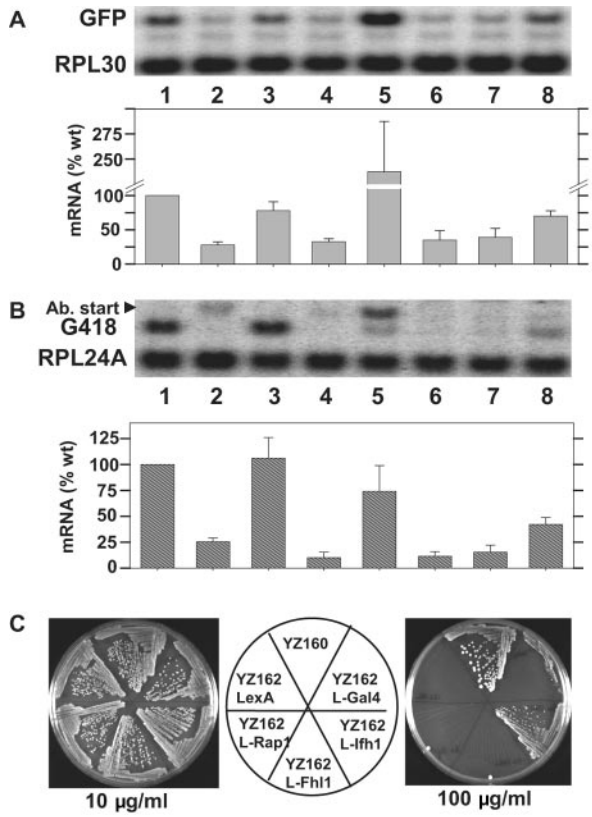

FIG. 3. Transcription and expression analysis of cells carrying integrated reporter constructs. (A and B) Northern analysis. All strains were grown at 30°C overnight and harvested when the cell density reached an optical density at 600 nm of 0.8 to 1.0. Total RNA was prepared and analyzed by Northern blotting. Lanes: 1, strain YZ170 containing the reporter with wild-type Rap1 sites; 2, strain YZ171 containing the reporter with deleted Rap1 sites; 3, strain YZ173 containing the reporter with reinserted Rap1 sites (RAP1*); 4 to 8, strain YZ172 containing the reporter with eight copies of LexA operators in the place of Rap1-binding sites, expressing LexA, LexA-Gal4(AD), LexA-Rap1, LexA-Fhl1, and LexA-Ifh1, respectively. The blot was probed with oligonucleotides JW61L for GFP and *RPL30* (A) and JW2258 for G418^r and *RPL24A* (B). Note that an internal control is built in since the same oligonucleotide was used for both wt and test genes. PhosphorImager data below each test gene were first normalized to the values of the corresponding RP gene (GFP against *RPL30* and G418^r against *RPL24A*) and then normalized to the wt values. The values for the G418^r transcript include molecules with the aberrant start site. Note that the band between L30 and GFP is the unspliced *RPL30* transcript, which can accumulate slightly (38). (C) G418 resistance. The strains indicated were streaked on yeast peptone dextrose plates containing 10 or 100 μg of G418/ml and incubated at 30°C for 2 days.

IFH1 was under GAL control. In both cases, the LexA fusion proteins supported growth in dextrose (Fig. 2B). Similarly, LexA-Fhl1 was able to rescue the slow-growth phenotype of an *fhl1*Δ strain (Fig. 2B, bottom). Therefore, fusion of the LexA DNA binding domain at the N terminus of Rap1, Fhl1, and Ifh1 does not interfere with their function.

LexA-Rap1 fails to activate. Total RNA was prepared from the strains with integrated constructs (Fig. 1) and analyzed by Northern blotting (43). A single oligonucleotide detects transcripts of both GFP and *RPL30* (Fig. 3A; see Materials and Methods), and another detects transcripts of both G418^r and *RPL24A* (Fig. 3B). Deletion of the Rap1-binding sites of the *RPL30*/*RPL24A* promoter leads to about 75% reduction in transcription (Fig. 3A and B, lane 2). The residual transcription is likely due to the T-rich elements found between the Rap1 sites and the initiation site in most RP genes, which contribute to RP promoter function (12). Interestingly, the residual transcription in the *RPL24A* direction starts about 200 bp upstream of the normal initiation site. Reinsertion of Rap1-binding sites restores transcription almost to its original level, indicating that the restriction sites interfere neither with the Rap1-binding sites nor with Rap1 function (Fig. 3A and B, lane 3).

Strain YZ172, with the integrated construct containing the LexA hybrid promoter (Fig. 1), was transformed with plasmids expressing the LexA DNA binding domain alone (Fig. 3A and B, lane 4) or the LexA fusion proteins, LexA-Gal4(AD), LexA-Rap1, LexA-Fhl1, and LexA-Ifh1 (lanes 5 to 8). As expected, in cells carrying the LexA hybrid promoter, expression of the LexA DNA binding domain alone has no effect on transcription (Fig. 3, lanes 4). On the other hand, expression of LexA-Gal4(AD) restores transcription in both directions (Fig. 3, lanes 5), although the site of initiation in the *RPL24A* direction is still mostly anomalous (Fig. 3B, lane 5). Nor does this transcript lead to G418 resistance (Fig. 3C), presumably because of aberrant splicing or aberrant translation initiation. These might lead to premature decay of the RNA, which would account for the apparent difference of LexA-Gal4(AD) activation in the two directions. On the other hand there is no reason to presume that Gal4(AD) will stimulate the same level of transcription in the two directions.

LexA-Rap1 fails to activate transcription (Fig. 3A and B, lane 6). Comparison of lanes 3 and 6 suggests that transcriptional activation of an RP gene requires that Rap1 bind DNA through its own DNA binding domain. Rap1 is known both to bend DNA (24) and to exclude nucleosomes (40). Presumably, one or both of these consequences of the binding of Rap1 are essential to recruit Fhl1 and Ifh1. The presence of the Rap1 polypeptide chain itself is insufficient.

LexA-Fhl1 also fails to activate transcription (Fig. 3A and B, lane 7). Thus, at the promoter of an RP gene, the presence of Fhl1, available to bind Ifh1, is not sufficient to drive transcription. This seems contradictory to previous work in which binding the FHA domain to DNA via the Gal4 binding domain led to recruitment of Ifh1 and activation of transcription (26, 33, 34). However, with such constructs no such activation occurs if the C-terminal region of Fhl1 is also present (34), as is the case in our experiment. Clearly, the large Fhl1 protein plays a more complex role in RP gene transcription than simply providing an FHA domain with which Ifh1 can interact.

Finally, LexA-Ifh1 alone does activate transcription (Fig. 3A and B, lane 8), although to a lesser extent than found in the wt gene. Furthermore, it restores transcriptional initiation in the *RPL24A* direction to the proper place, as confirmed by the resistance of the cells to G418 (Fig. 3C). This result suggests that Ifh1 is a transcriptional (co)activator for RP genes and that it is at least partly responsible for selection of the proper initiation site. The basis for the selection of the initiation site (often sites) in yeast is obscure. RP genes are said not to use a canonical TATA box (16), and the region upstream is very AT rich.

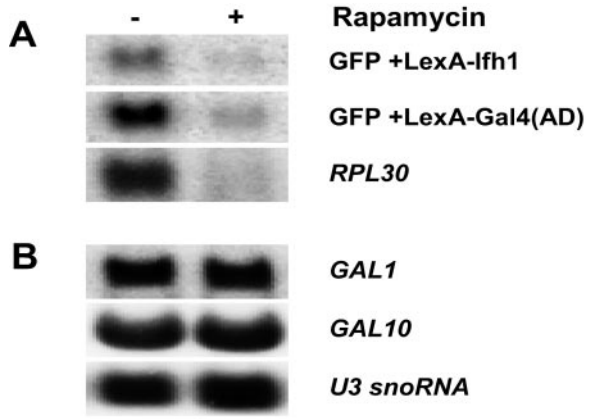

FIG. 4. Transcription activated by LexA-Ifh1 and LexA-Gal4(AD) is repressed by rapamycin. (A) Strain YZ172, expressing LexA-Ifh1 or LexA-Gal4(AD), was grown to log phase in SC dropout medium, and a sample of each culture was harvested. Rapamycin was added to the remainder at a final concentration of 200 ng/ml. After 60 min the cultures were harvested. Total RNA was prepared and subjected to Northern analysis. GFP and *RPL30* were probed with oligonucleotide JW61L. PhosphorImager quantitation showed that after treatment with rapamycin the level of GFP transcripts was reduced by approximately 80% for both strains and the level of *RPL30* transcripts by about 90%. (B) W303 was grown in YPGal; *GAL1* and *GAL10* transcript levels of cultures were analyzed before and after treatment with 200 mg/ml rapamycin for 60 min. The transcript level of *GAL1* or *GAL10* is barely affected.

Transcription under the control of the LexA hybrid promoter is repressed by rapamycin. Loss of Ifh1 from an RP gene has been implicated as the proximal cause for the repression of transcription by rapamycin (26, 33, 34, 39). In addition, Martin et al. showed that Crf1, a protein resembling Ifh1, binds to Fhl1 at RP promoters following rapamycin treatment and is required for their repression (26). Remarkably, treatment of cells with rapamycin represses not only *RPL30* but also the GFP transcript activated either by LexA-Ifh1 or by LexA-Gal4(AD) (Fig. 4A). Note that rapamycin has no effect on normal transcription driven by Gal4 (Fig. 4B). This result is consistent with our previous finding that rapamycin inhibits the very limited transcription of RP genes in cells lacking Fhl1 and Ifh1 (33). These observations suggest that the TOR pathway is required not only to promote the interaction of Ifh1 with Fhl1 but also to facilitate the residual transcription that occurs independently of the Rap1-Fhl1-Ifh1 transcription complex.

To ask whether the Crf1 repressor might be involved in the rapamycin effect described above, we first examined the consequence of *CRF1* deletion in an otherwise wild-type background. Surprisingly, we found that *crf1*Δ has no measurable effect on the repression at the three different RP genes tested (*RPL9A*, *RPL30*, and *RPL37A*) in our W303 strain background (Fig. 5). We confirmed the role of Crf1 in repression of the same genes following rapamycin treatment in strain TB50 (Fig. 5). Consistent with the absence of a role for Crf1 at RP gene promoters in W303, we have consistently failed to detect the protein at RP genes by ChIP (data not shown). We thus conclude that the effect of rapamycin on transcription driven by LexA-Gal4(AD) is unlikely to require Crf1. We have crossed W303 and TB50 in an attempt to characterize the genetic basis

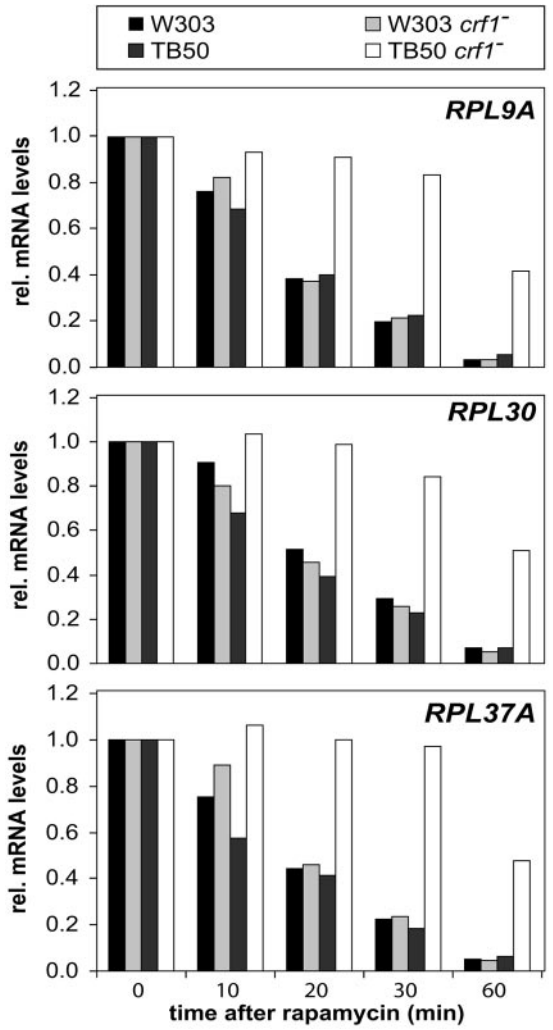

FIG. 5. The effects of Crf1 are strain specific. The rapamycin response at RP genes in W303, unlike that in TB50, is independent of Crf1. Log-phase cells were treated with rapamycin (200 ng/ml), and mRNA levels of the indicated RP genes were measured at 0, 10, 20, 30, and 60 min as described previously (34).

of the difference in Crf1 function in these two strains. However, the heterozygous diploid displayed an intermediate phenotype by transcript analysis, and the behavior of haploid segregants suggested that the difference is not due to a single gene.

Rap1-RPG box interaction is necessary to recruit Fhl1 and Ifh1. To determine whether the binding of Rap1, Fhl1, and Ifh1 to the modified promoters of *RPL24A*/*RPL30* was altered, we performed ChIP analysis on strains carrying Fhl1 and Ifh1 C-terminally tagged with HA_3 and Myc_{13}, respectively (Table 1; Fig. 6). As controls, we also tested their binding to the *ACT1*, *PGK1*, and *RPL11A* genes. None of the three proteins are found at the *ACT1* gene (19, 33), which was used as a negative control. As shown in Fig. 6, Rap1 is present at *PGK1* but Fhl1 and Ifh1 are not. This is expected since the glycolytic genes are activated by Gcr1 in collaboration with Rap1 (8), rather than by Fhl1 and Ifh1 (19, 34). At the promoters of both *RPL11A* and *RPL24A*/*RPL30* all three proteins are present, as expected from previous work (19, 33, 34). Deletion of the Rap1

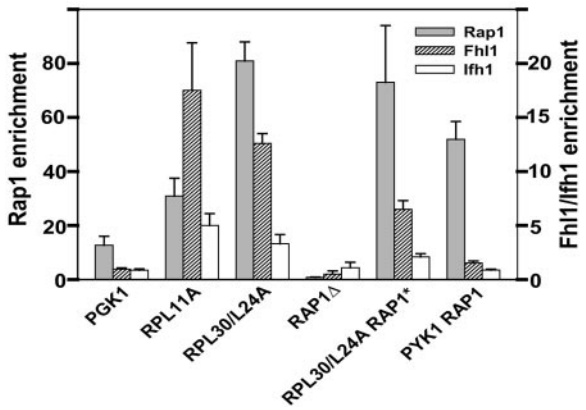

FIG. 6. Rap1, Fhl1, and Ifh1 occupancies at the indicated promoters. ChIP was performed using anti-Rap1, anti-HA, or anti-Myc antibodies on strains YZ171 and YZ173 (with HA-tagged Fhl1 and Myc-tagged Ifh1). Following ChIP, real-time PCR was performed on total chromatin (input) and the immunoprecipitated DNA with primers specific for the promoters of the indicated RP genes. Primers specific for the promoter of *ACT1* were used as a negative control (19, 33), against which other genes were normalized to calculate enrichment (*n*-fold). Gene-specific PCR primers were designed such that it is possible to distinguish wild type (*RPL24A*/*L30*) from the promoter without the Rap1 sites (Table 2). Note the different scales.

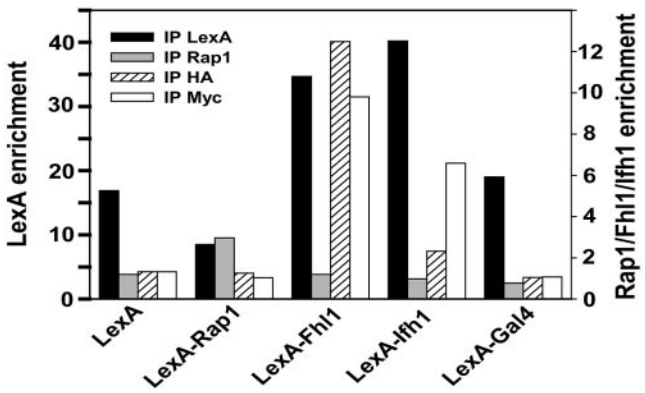

FIG. 7. Tethering Rap1 to the LexA hybrid promoter fails to restore binding of Fhl1 or Ifh1. ChIP was performed using anti-LexA, anti-Rap1, anti-HA, or anti-Myc antibodies on strain YZ172 (with the LexA hybrid promoter [Fig. 1] and carrying tagged proteins Fhl1-HA_3 and Ifh1-Myc_9), expressing LexA or one of several LexA fusion proteins as indicated. Primers specific for the LexA hybrid promoter were designed for real-time PCR analysis. Note the different scales.

sites abolishes binding not only of Rap1 but also of Fhl1 and Ifh1, indicating that Rap1 binding is essential for the recruitment of both. Reinsertion of the Rap1 sites restores binding of all three, but to a somewhat lesser extent. We cannot tell if this is due to the introduction of the restriction sites or is an artifact due to the specific oligonucleotide pair needed to distinguish the test gene from the wt gene. On the other hand, replacing the Rap1-binding sites of *RPL24A*/*L30* with that of *PYK1*, a glycolytic enzyme gene, leads to strong recruitment of Rap1 but not of either Fhl1 or of Ifh1 (Fig. 6). These results show once again that context is critical to the selection of the cofactors of Rap1 at individual promoters.

Consequences of the binding of a LexA fusion protein. We next asked whether the failure of LexA-Rap1 to activate transcription from the LexA binding sites (Fig. 3, lanes 6) is due to its inability to recruit Fhl1 and/or Ifh1. In the presence of LexA alone none of the three proteins were found (Fig. 7). In the presence of LexA-GAL4(AD), again Rap1, Fhl1, and Ifh1 were not present. LexA-Gal4(AD) was present, as predicted by the vigorous transcription that it activated (Fig. 3, lanes 5).

In cells expressing both Rap1 and LexA-Rap1, ChIP using anti-LexA antibody, recognizing only the fusion protein, or anti-Rap1 antibody, recognizing both, shows the presence of LexA-Rap1 at the LexA-op site (Fig. 7). (Note that our anti-Rap1 antibody poorly recognizes the LexA-Rap1 fusion protein [data not shown].) Yet, almost no Fhl1 or Ifh1 is associated with the LexA-Rap1 bound at the LexA-op sites. This result implies that it is not the presence of the Rap1 protein but the binding of Rap1 to its sites that somehow modifies the DNA, or the chromatin, to induce the association of Fhl1 and Ifh1 As noted above (Fig. 6), even binding of Rap1 through a site from the *PYK1* promoter is not sufficient for Fhl1 and Ifh1 recruitment.

LexA-Fhl1 binds to the LexA-op sites and appears to recruit Ifh1, but not Rap1 (Fig. 7). This result is consistent with the observation that Fhl1 is able to coimmunoprecipitate Ifh1 (reference 33 and data not shown). Nevertheless, the recruitment of Ifh1 by LexA-Fhl1 does not activate transcription (Fig. 3A and B, lane 7), suggesting that a particular geometry of the DNA-Fhl1-Ifh1 complex is required. Although this could involve the binding of Fhl1 to the DNA, we have found that the putative DNA binding "forkhead" domain of Fhl1 is of little importance for cell growth (32). An intriguing observation is that the endogenous Fhl1-HA_3 is also recruited to the LexA-op sites. This result suggests that Fhl1 forms a dimer in vivo, although we have been unable to confirm this with coimmunoprecipitation experiments on extracts (data not shown). The interaction may be transient, observable only in extracts of formaldehyde-fixed cells.

LexA-Ifh1 binds to the LexA-op site (Fig. 7) and in this case brings about substantial transcription (Fig. 3, lanes 8). This result suggests that Ifh1 can act as a transcriptional activator, and conversely, that neither Rap1 nor Fhl1 itself is a transcriptional activator of RP genes. However, comparison of the levels of LexA-Gal4(AD) and LexA-Ifh1 at the promoter with the levels of transcription by each suggests that Ifh1 by itself is a rather weak activator. Again, the endogenous Ifh1-Myc_{13} is also present at the LexA-op promoter, suggesting possible dimerization.

Rap1 and Fhl1/Ifh1 are at distinct locations in RP gene promoters. Previously, mapping of Fhl1 and Rap1-binding sites across three RP promoters, *RPL12A*, *RPS11B*, and *RPL40A*, suggested that there are promoter-specific differences in the relative locations of these proteins (39). Fhl1 was found either at the same location as Rap1 or between Rap1 and the transcription start site.

To determine more precisely the locations of Rap1, Fhl1, and Ifh1 at RP promoters, we performed high-resolution ChIP on three intergenic regions at which RP genes were head to head either with another RP gene (*RPL24A*/*L30* and *RPS22A*/*L39*) or with a non-RP gene (*PRE2*/*RPL11A)*, using primer pairs covering every 100 bp of the intergenic region (Fig. 8). In

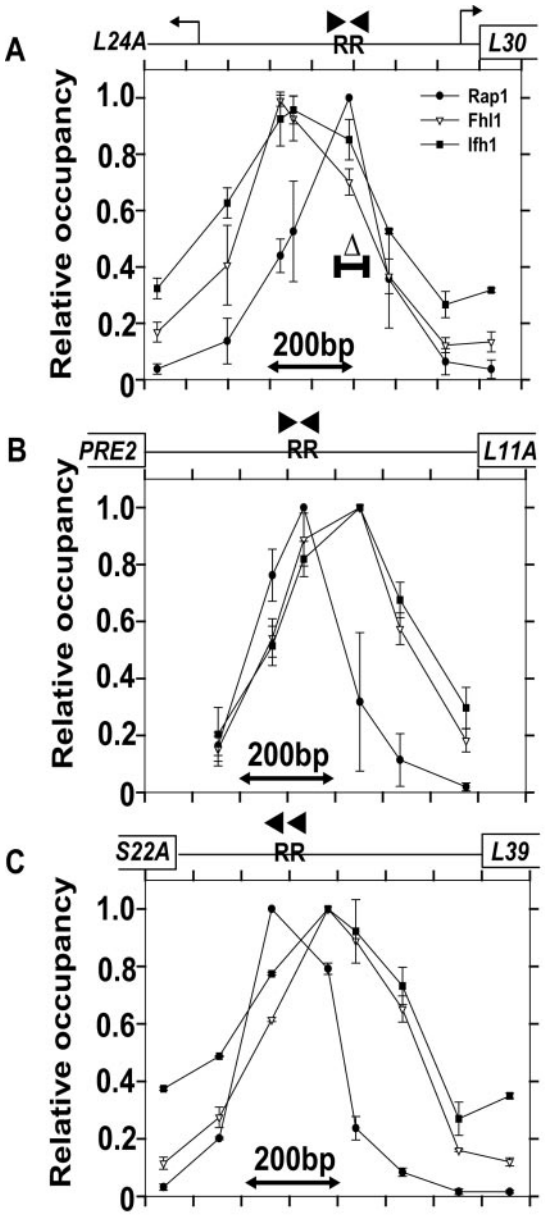

FIG. 8. Fhl1 and Ifh1 coincide but bind at a location distinct from Rap1 at RP gene promoters. Primers used for real-time PCR analysis of ChIP DNA were designed for every 100-bp region of the RP gene promoters. The position of the middle of the PCR products was taken for the *x* coordinates. The figure shows relative occupancy of Rap1, Fhl1, and Ifh1 across the promoters of *RPL24A*/*L30* (A), *PRE2*/*RPL11A* (B), and *RPS22A*/*RPL39* (C), normalized to the highest occupancy value. The positions of predicted Rap1 DNA binding sites are indicated above each graph by boxes with R's, and the orientation of the site is indicated by an arrowhead (17). The extent of the deletion shown in Fig. 1 is indicated. The positions of the transcription initiation sites are indicated by arrows, when known. The *x* coordinate is marked every 100 bp, and the beginnings of the ORFs are indicated by boxes. (The ORF of *RPL24A* starts 490 nucleotides downstream of the transcription initiation site due to an intervening intron.) Error bars show the standard deviations of the means.

this case, the chromatin was sheared to a mean size of ~200 bp. The peak of Rap1 binding is sharply defined, directly over the predicted Rap1-binding sites (17). However, Fh1l and Ifh1 are distinct from Rap1 at these RP gene promoters. The location of Ifh1 coincides with that of Fhl1, consistent with the notion that Ifh1 is recruited to the RP promoter through its interaction with Fhl1 (33, 34, 39). In both cases of head-to-head RP genes, while the Rap1 sites are asymmetrical with respect to transcription initiation, Fhl1 and Ifh1 are located in a single peak approximately equidistant from the initiation sites. This result suggests that there is no directionality to their function. Yet, in the case of the *RPL11A*/*PRE2* pair, the transcription of *PRE2* is regulated entirely differently from that of *RPL11A* (11).

The key conclusion from Fig. 8 is that Ifh1 and Fhl1 are found at the same site on RP promoters, rather distant from the site of transcription initiation, and clearly 100 to 200 bp distinct from the site of Rap1 binding. In particular, Fhl1 and Ifh1 need not necessarily lie between Rap1 and the initiation site.

Employing the same chromatin preparations, we asked about the presence of nucleosomes at the RP promoters, using antibody directed against the unmodified C terminus of histone H3 (Fig. 9). It is evident that the Rap1 sites are almost completely clear of nucleosomes, as suggested by the genome-wide data recently published (3, 30, 41) and the observation that RP gene promoters are particularly deficient in Htz1, an H2A variant implicated in regulation (42).

DISCUSSION

Based on recent work, a simple model for the transcription of RP genes is that Rap1 recruits Fhl1, which in turn recruits the transcriptional activator Ifh1. Regulation of transcription involves the control of the Fhl1-Ifh1 interaction (26, 33, 34, 39). Using a minimally modified promoter driving the transcription of two RP genes in head-to-head configuration, we set out to examine in more detail the role of Rap1 in active transcription of the RP genes and to evaluate the degree to which the simplest form of the model can account for the observations.

Indeed, deletion of Rap1 sites from the promoter of a pair of RP genes (Fig. 1) leads to a major loss of transcription (Fig. 3) as well as to the loss of Fhl1 and Ifh1 binding (Fig. 6). Reintroduction of the Rap1 sites restores transcription and the binding of all three factors. However, replacing the native Rap1 sites with the Rap1 site from the glycolytic gene *PYK1* restores the binding of neither Fhl1 nor Ifh1 (Fig. 6), although some transcription ensues, in only one direction, possibly through the recruitment of Gcr1 (data not shown). This result mirrors the published genome-wide analyses that found neither Fhl1 (19) nor Ifh1 (34) at glycolytic genes, where Gcr1 and Gcr2, rather than Fhl1 and Ifh1, act as coactivators (also Fig. 6, data for *PGK1*) (8, 37).

Since Rap1 operates at so many genes, in both positive and negative ways (see the introduction), the sequence elements that designate which coactivators or corepressors associate with Rap1 at a particular locus are of key importance but have yet to be clearly delineated. Although some attempts have been made in silico, by examining the relationship between sequence and coregulation (2, 39), the proposed sequences are not found at every RP gene. While there are close matches to the IFHL sequence element suggested by Wade et al. (39) within both the *RPL24A-RPL30* and the *RPS22A-RPL39* promoters, right at the peak of Fhl1/Ifh1 binding, there is none

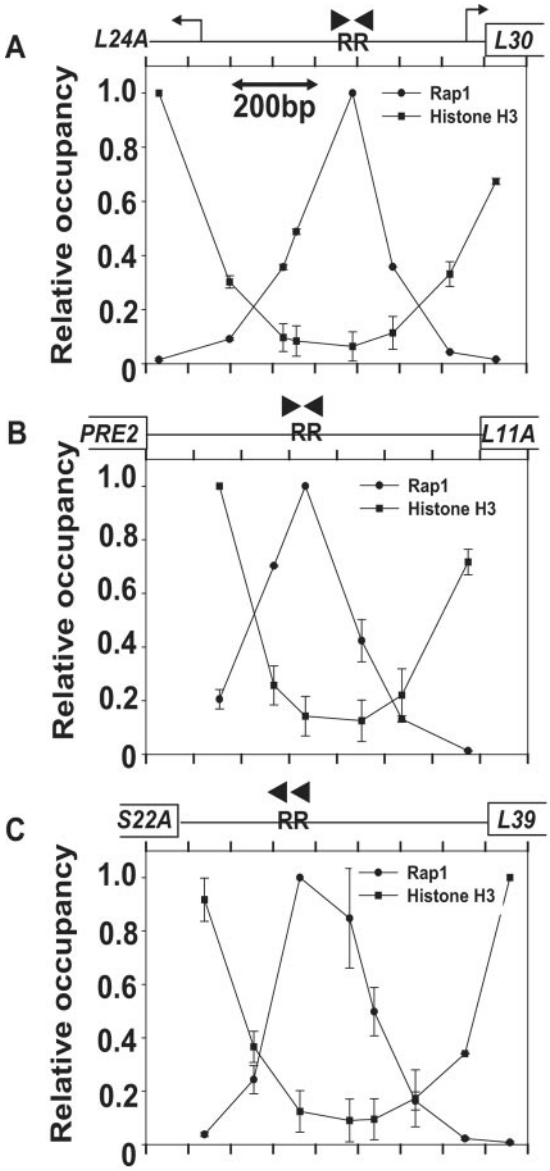

FIG. 9. Rap1 excludes nucleosomes from RP promoters. ChIP analysis was performed using an anti-histone H3 C-terminal region antibody (ab1791; Abcam) and anti-Rap1 antibody. The same primer pairs were used as in Fig. 8. The figure shows relative occupancy of Rap1 and histone H3 across the promoters of *RPL24A*/*L30* (A), *PRE2*/*RPL11A* (B), and *RPS22A*/*L39* (C), normalized to the highest occupancy value. The *x* coordinate is marked every 100 bp, and the beginnings of the ORFs are indicated by boxes.

upstream of the *RPL11A* gene. Thus, while the IFHL motif is promising, it seems unlikely to be key to the recruitment of Fhl1 and Ifh1.

A useful insight comes from our observation that for the recruitment of Fhl1 and Ifh1 to a RP promoter, Rap1 must bind directly to its sites on the DNA (Fig. 6). The presence of the Rap1 polypeptide chain, held at the promoter by LexA, is insufficient. A primary result of the direct binding of Rap1 to DNA is undoubtedly to clear nucleosomes from the region (28, 40). Indeed, nucleosomes are almost entirely absent from the Rap1 sites in front of RP genes (Fig. 9) (4, 18, 30). Nevertheless, we remain ignorant about the specificity with which Rap1 selects the appropriate cofactors at any given class of genes.

The importance of Rap1 in bringing about an arrangement of chromatin hospitable to Fhl1 and Ifh1 is demonstrated by the observation that deletion of the Rap1-binding sites leads to loss of the two proteins (Fig. 7), even though the region of the promoter with which they are associated, some 100 to 200 bp distant, remains intact (Fig. 8). Although Fhl1 has a domain that is related to the DNA binding domain of the *Drosophila melanogaster* Forkhead protein, Fhl1 does not bind DNA in vitro, nor is the Forkhead domain important for RP gene transcription or cell growth (33). Thus, the binding of Fhl1, and by implication Ifh1, to a normal RP promoter is likely to be indirect, either secondary to or facilitated by another protein. Is this simply Rap1 itself? If so, what determines the specificity?

Two observations raise questions about the arrangement of factors necessary to drive transcription at the RP genes. One is that there seems to be a single site on the DNA for the association of Ifh1 and Fhl1, even when they are driving two RP genes (Fig. 8). Previous work identified Fhl1 as coincident with Rap1 or slightly proximal to the transcription initiation site (39), as we see for *RPL11A* (Fig. 8B). However, in the cases where two RP genes are driven from the same promoter, Fhl1 and Ifh1 are located in a single, albeit relatively broad, site asymmetrical with respect to the Rap1 sites, yet approximately equidistant from the two initiation sites. Transcriptome data suggest that both genes are transcribed to roughly the same extent (15). Thus, Ifh1 appears to be able to drive transcription from a position distal to Rap1. How can it overcome the barrier that the two Rap1 molecules seem to impose on chromatin structure?

The other observation is that although LexA-Fhl1 recruits Ifh1 to the promoter, no transcription ensues. There are two possible explanations. One is that the geometry of the site is incorrect in this configuration, i.e., that Fhl1 must hold Ifh1 in just the right orientation and does not do so as a LexA fusion. However, this seems unlikely, as the presumably flexible LexA-Ifh1 is able to drive transcription in both directions. Another possibility is that the eight LexA sites led to congestion on the promoter, such that the activation region of Ifh1 was unable to address the transcription site. However, when the experiments of Fig. 3 were repeated using only two LexA sites, essentially identical results were obtained, except that the levels of transcripts of both L24A-G418^r and L30-GFP were substantially reduced. Both the aberrant transcription initiation by LexA-Gal4(AD) and the correct one by LexA-Ifh1 were reproduced. An alternative possibility is that, although the FHA domain of Fhl1 can recruit Ifh1 to promote transcription of a test gene (26, 34), Fhl1 in its intact form acts as a repressor, as was originally suggested based on the observation that deletion of *FHL1* restored the viability (although barely) to a strain without *IFH1* (5). Thus, we must consider Fhl1 as more than a platform to present an FHA domain with which Ifh1 can interact. Other parts of Fhl1 may affect the rate of transcription of the RP gene or may transduce other regulatory signals.

A final point that casts some doubt on the simple model described above is that rapamycin inhibits the transcription driven by LexA-Ifh1 as well as by LexA-Gal4(AD). Thus, the scenario in which the mechanism of action of rapamycin is either to modify Ifh1 (33, 34) or to cause Crf1 to move from

the cytoplasm to the nucleus to replace Ifh1 (26) seems inadequate. Rather, our data suggest that although rapamycin does act to reduce the interaction of Ifh1 with Fhl1, it must also work in some way independently of Fhl1 and Ifh1, as suggested previously for cells devoid of both (33). Further underscoring the complexity of the rapamycin response, as well as its variability, we show that the Crf1 repressor has no apparent role in our strain background. Thus, Ifh1 leaves RP gene promoters in the absence of competition by Crf1 in W303 (strain YSS120, Table 1), indicating that the rapamycin response at the level of Fhl1 and Ifh1 displays remarkable strain variability. An understanding of the genetic basis of this difference might reveal interesting features of the TOR pathway as it impinges upon RP genes.

Another factor implicated in the regulation of RP gene transcription is Sfp1 (25). Although ChIP analysis showed little binding of Sfp1 to the L24A-L30 intergenic region (19, 25), bioinformatics approaches suggested that both *RPL24A* and *RPL30* could be down-regulated by the transfer of Sfp1 from the nucleus to the cytoplasm caused by the presence of rapamycin and other repressive conditions (25). Therefore, it seems at least possible, if not likely, that the transcription caused by LexA-Gal4 and LexA-Ifh1 was repressed by rapamycin through its effect on Sfp1. Based on the results in Fig. 4 and on our results previously reported that rapamycin represses the residual transcription of RP genes in cells without Fhl1 (33), we suggest that the action of Sfp1 on RP gene transcription is independent of the Rap1-Fhl1-Ifh1 transcriptional complex and may indeed involve other factors or other sequences than the Rap1-binding sites.

In summary, we have shown that direct binding of Rap1 to the promoter of RP genes excludes nucleosomes and recruits Fhl1 and Ifh1 to drive transcription, consistent with the simple model presented above. However, a number of observations suggest that the simple model is insufficient. Recruitment of Ifh1 by LexA-Fhl1 does not drive transcription, suggesting either that the geometry of the arrangement is important or that Fhl1 can act as a repressor. The binding sites of Fhl1 and Ifh1 are coincident but at some distance from the Rap1 sites, which themselves are asymmetrically arranged in the two head-to-head pairs of RP genes that we have examined. Rapamycin inhibits transcription of RP genes that are driven by the Gal4(AD), suggesting that something beyond the interaction of Fhl1 and Ifh1 is responsive to the TOR pathway.

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We dedicate this paper to the memory of Stephan Schawalder, a most promising young scientist.

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