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Genetic Analysis of Histone H4: Essential Role of Lysines Subject to Reversible Acetylation

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The nucleosome is the fundamental unit of assembly of the chromosome and reversible modifications of the histones have been suggested to be important in many aspects of nucleosome function. The structure-function relations of the amino-terminal domain of yeast histone H4 were examined by the creation of directed point mutations. The four lysines subject to reversible acetylation were essential for histone function as the substitution of arginine or asparagine at these four positions was lethal. No single lysine residue was completely essential since arginine substitutions at each position were viable, although several of these mutants were slower in completing DNA replication. The simultaneous substitution of glutamine for the four lysine residues was viable but conferred several phenotypes including mating sterility, slow progression through the G_2/M period of the division cycle, and temperature-sensitive growth, as well as a prolonged period of DNA replication. These results provide genetic proof for the roles of the H4 amino-terminal domain lysines in gene expression, replication, and nuclear division.

THE NH_2-terminal domain of histone H4 has been highly conserved throughout evolution. The first 20 amino acids of yeast and mammalian histone H4 are identical, suggesting essential functions for this region of the protein. The NH_2-terminal domain includes the sites for a variety of posttranslational modifications including methylation, phosphorylation, and acetylation (*1*). In yeast, as in other eukaryotes, four lysine residues at positions 5, 8, 12, and 16 are subject to reversible acetylation at their ϵ-amino groups (*2*). Changes in lysine acetylation states have been correlated with the onset of a variety of nuclear functions, including transcription, replication, chromatin assembly, and spermatogenesis (*1, 3*). These acetylation changes are presumed to be critical in effecting the dynamic alterations of histone-DNA and histone-protein interactions required for function. Therefore, it is perhaps surprising that the wild-type yeast histone H4 genes can be replaced with alleles that lack the sequences encoding amino acids 4 to 28 (*4*) or 2 to 26 (*5*). Cells with these deletion alleles as their sole source of histone H4, though viable, suffer a variety of defects including a slower growth rate, derepression of the silent mating-type genes resulting in phenotypic sterility (*4, 5*), and temperature-sensitive growth (*5*).

One interpretation of the unexpected viability of the NH_2-terminal deletion mutants is that the four lysine residues do not provide an essential biological function and that changes in their acetylation states are merely coincidental. To test this hypothesis, an allele of the yeast histone H4 gene was constructed (*6*) in which all four lysine codons at amino acid positions 5, 8, 12, and 16 were changed to arginine codons (*hhf1-9*). The functions of this allele, as well as the others used in this study, were assayed by the complementation of histone gene deletions in yeast. The histone H3 and H4 genes in *Saccharomyces cerevisiae* are arranged as divergently transcribed gene pairs with one H3 gene and one H4 gene sharing a common promoter region. To avoid disrupting the normal regulation of the genes and to maintain the stoichiometry of H3 to H4 histone, we engineered all constructs as H3-H4 gene pairs. The haploid genome contains two nonallelic sets of H3-H4 gene pairs (*7*). The copy-I H3 (*HHT1*) and H4 (*HHF1*) genes make up the first set, while the copy-II H3 (*HHT2*) and H4 (*HHF2*) genes make up the second unlinked set. Either set of genes is sufficient for cell growth, but simultaneous deletion of both loci is lethal (*8*). The *hhf1-9* allele together with a wild-type histone H3 gene (*HHT1*) were used to complement deletions of both chromosomal gene sets by two different protocols (*9*). In the first procedure, we attempted to rescue a spore colony deleted for both chromosomal copies of the H3-H4 gene sets (Fig. 1A); such colonies are recognized by their nonsectoring white phenotype and sensitivity to 5-fluoroorotic acid (5-FOA). Although a control plasmid carrying the wild-type H4 gene could efficiently rescue the chromosomal deletion segregants, the *hhf1-9* allele failed to give viable colonies in both tetrad dissections and random spore screens. We also tested *hhf1-9* using a high copy number yeast vector in case it could provide minimal function at higher gene dosage. These derivatives also failed to give colonies.

To eliminate the possibility that *hhf1-9* was only defective in spore germination, we tested it in a "plasmid shuffle" assay (*10*) (Fig. 1B). A control plasmid containing the wild-type H4 gene produced colonies at high efficiency in this test, but the *hhf1-9* allele did not produce colonies, indicating that the mutant was not viable. Since cells carrying the *hhf1-9* allele grew normally in the presence of either a chromosomal or a plasmid copy of the wild-type H4 gene, the mutations in *hhf1-9* must confer a recessive loss of function rather than a dominant lethality. Thus, one or more of the lysine residues at positions 5, 8, 12, and 16 provide an essential function when the NH_2-terminal domain is present intact and cannot be mutated to arginine without the loss of this function.

These results suggested that the inviability of *hhf1-9* might be caused by the continuous presence of positively charged arginine side chains. According to this model, the presence of positive charges at positions 5, 8, 12, and 16 would be lethal at some stage of the cell cycle; in the wild-type gene, the charged lysine residues would be neutralized by acetylation, and in the NH_2-terminal deletion mutation, the positive charges would be missing completely. This model made two predictions: that replacement of the four lysines with unchanged amino acids would produce a viable phenotype, and that the mutant would mimic the behavior of the NH_2-terminal deletion mutants.

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To test these predictions, we constructed an allele in which the four lysine codons were simultaneously changed to glutamine codons (*hhf1-10*). When tested in the plasmid shuffle assay, *hhf1-10* was capable of replacing the wild-type H4 gene, in agreement with the first prediction of the model. The second prediction was tested by comparing the properties of *hhf1-10* with those of the NH_2-terminal deletion allele *hhf1-8* (*5*). Three phenotypes produced by the H4 NH_2-terminal deletion were examined: (i) defective mating ability, (ii) increased generation time (*4*, *5*), and (iii) temperature-sensitive growth (*5*). The results of quantitative mating assays on isogenic strains differing only in their H4 alleles are presented in Table 1. Strains with either chromosomal or plasmid copies of the wild-type H4 genes had normal mating efficiencies of 50% or better. In contrast, *hhf1-8* and *hhf1-10* were sterile, mating with efficiencies of less than 0.01%. As with the NH_2-terminal deletion mutants, *hhf1-10* strains had a generation time that was about 70% longer than isogenic strains carrying the wild-type H4 gene (see Table 1). This slower division cycle was predominantly the result of a longer G_2 period, also characteristic of the NH_2-terminal deletion mutants (*4*, *5*). Finally, although cells expressing the wild-type H4 plasmid grew normally at both 28° and 37°C, neither *hhf1-8* nor *hhf1-10* strains were

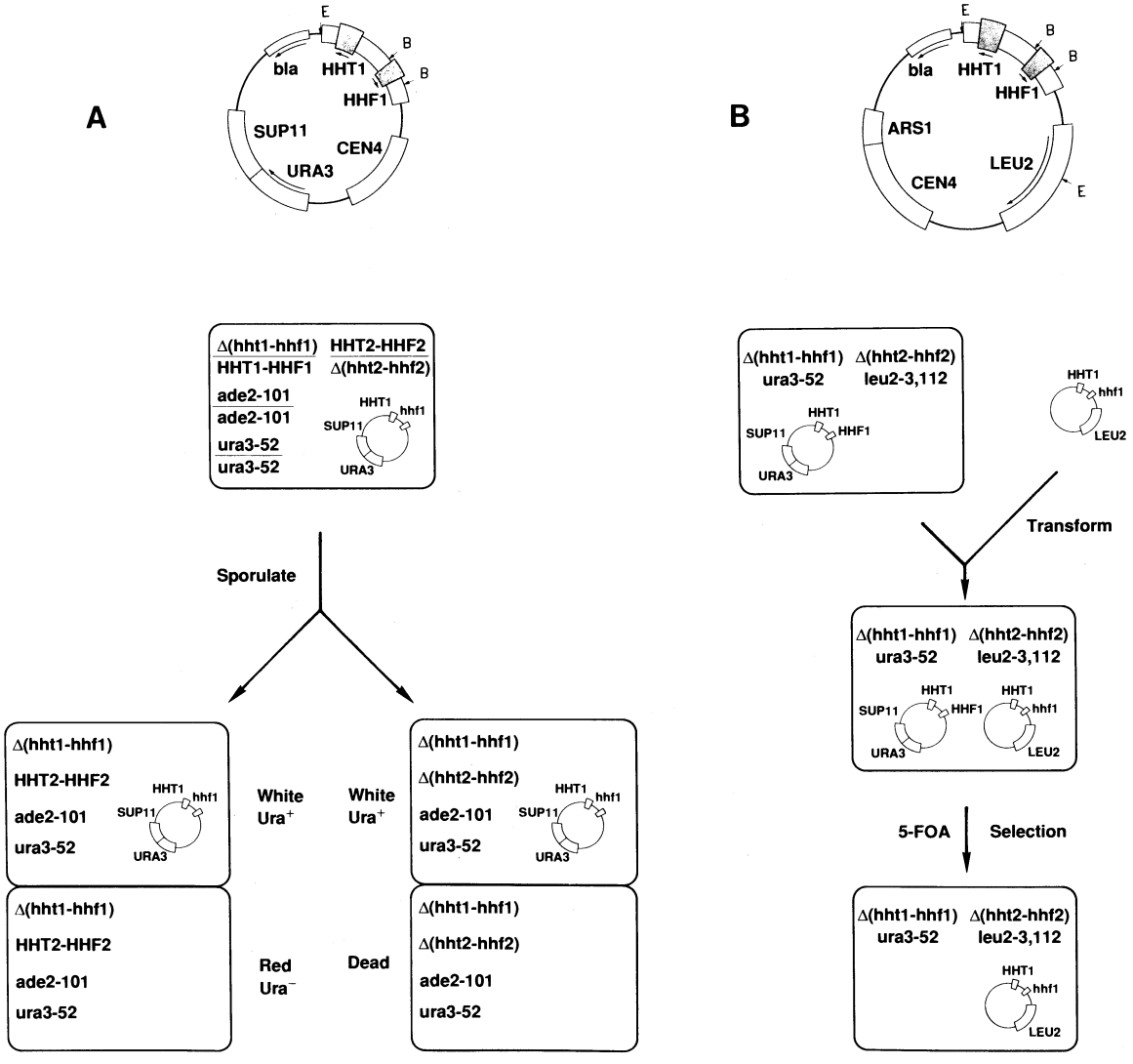

Fig. 1. Two protocols were used to test histone H4 alleles in vivo. The assays relied on the ability of specialized yeast transformation plasmids (*9*) to rescue a strain deleted for all chromosomal copies of the H3 and H4 genes. The H4 alleles (Table 1) were subcloned into the Bam HI site for testing. (**A**) The protocol for plasmid rescue by meiotic segregation. The starting host strain was a diploid hemizygous for both histone H3-H4 gene sets and homozygous for *ura3-52* and *ade2-101*. The *ade2-101* ochre mutation causes the accumulation of a red pigment and the formation of red colonies. This host was transformed with the test H4 gene (*hhf1*) on a plasmid containing *HHT1*, *URA3*, *SUP11*, and *CEN4*. The diploid transformant was then sporulated to give haploid spore segregants. The left pathway illustrates the case in which one or more of the chromosomal H3-H4 loci are inherited. The *SUP11* ochre suppressor on the plasmid suppresses the adenine mutation in the transformants and gives white colonies. If the plasmid is present, the cells are white, Ura^+, and sensitive to 5-FOA, a drug that prevents growth of cells expressing the wild-type *URA3* gene (*23*). If the plasmid is lost during nonselective mitotic growth, the cells are viable, red, Ura^-, and resistant to 5-FOA. In practice, these meiotic segregants produced red and white sectored colonies that were capable of growing in the presence of 5-FOA. The right pathway of (A) illustrates the case in which both H3-H4 deletion loci were inherited. Under nonselective growth, loss of the plasmid is lethal. Therefore, these segregants were identified as nonsectoring white colonies sensitive to 5-FOA. (**B**) The design of the plasmid shuffle protocol (*10*). The starting yeast host was a haploid deleted for both chromosomal H3-H4 loci and rescued by the wild-type H3 and H4 genes carried on the *URA3/CEN4* plasmid as described in (A). This strain was then transformed with a second *LEU2/CEN4* plasmid containing the wild-type H3 gene (*HHT1*) and the test allele of the H4 gene (*hhf1*). The transformants were then plated on 5-FOA medium to select for loss of the original *URA3* plasmid. The histone and plasmid genotypes for isolates from both protocols were confirmed by Southern blot analysis of purified DNA.

able to form colonies at 37°C. Thus, these assays show that cells bearing the *hhf1-10* allele are viable and share major phenotypic traits with those carrying *hhf1-8*.

Cells dependent on the *hhf1-10* allele manifested a number of other phenotypic defects. Most noticeably, *hhf1-10* cells were unusually large and polydisperse in size. The distribution of cell volumes for the *hhf1-10* strain was skewed toward larger cell sizes (*12*); the mean cell volume of *hhf1-10* was 115 femtoliters (fl), while the individual averages for all the other strains were approximately 54 fl. The *hhf1-10* cells also displayed abnormal morphologies (*13*) (Fig. 2). Elongated cells and buds with partial constrictions along their length were found at a frequency of 10 to 15%, and these often had multiple nuclei per cell. Such cells were not found in isogenic strains carrying either the wild-type H4 gene or any of the other H4 point mutations. These results show that *hhf1-10* is sufficient for viability but lacks complete H4 function.

To examine whether other uncharged or neutral polar amino acids were capable of replacing the NH_2-terminal domain lysines, we made an allele (*hhf1-11*) in which the four lysines were simultaneously replaced with asparagines. Cells in which the *hhf1-11* allele replaced the wild-type H4 gene were not viable. Therefore, reversible positive-charge neutralization cannot be the only factor required for the function of the NH_2-terminal domain in vivo.

Since cells were unable to survive when forced to depend on *hhf1-9*, the allele with the four arginine substitutions, we sought to identify which of the individual lysine residues were required for vital functions. Since previous work in *Tetrahymena* and *Physarum* had suggested that acetylation of Lys^5 and the pairs of residues Lys^5-Lys^8 and Lys^5-Lys^{12} would be critical for function (*14*), we first made an allele (*hhf1-13*) in which the lysine at position 5 was replaced by an arginine. When assayed by plasmid shuffle, this allele was able to replace the wild-type H4 gene and gave cells that grew normally, were not temperature sensitive, and mated at wild-type frequencies (Table 1). To date, no mutant phenotype has been detected for strains dependent on the *hhf1-13* allele.

Next, double-substitution alleles were constructed to examine directly the function of the two predominant acetylation patterns observed in *Tetrahymena*. In the *hhf1-14* allele, arginines were substituted for Lys^5 and Lys^8, and in the *hhf1-15* allele arginines were substituted for Lys^5 and Lys^{12}. Both of these alleles could replace the wild-type H4 gene, giving strains that were not temperature-sensitive and that mated at wild-type frequencies. This left the possibility that the lysine at residue 16 was exclusively responsible for the lethality of *hhf1-9*. To test this, we altered the lysine codon for residue 16 to give an arginine codon in *hhf1-21*. This allele was also able to replace the wild-type H4 gene, and the isolates were not temperature-sensitive for growth. However, unlike the previous set of lysine to arginine mutations, *hhf1-21* had a slight but reproducible defect in mating efficiency. In quantitative assays the mating frequency for these cells was only about 24 to 34%, one-half the frequency of isogenic strains carrying wild-type H4 genes (Table 1). Thus, the individual lysine residues in the NH_2-terminal domain of histone H4 are dispensable for functions essential for viability. Therefore, it is unlikely that specific acetylation of either the Lys^5-Lys^8 or the Lys^5-Lys^{12} amino acid pair is obligatory for essential histone H4 functions in vivo in *S. cerevisiae*.

The mating defect in cells containing *hhf1-21*, the arginine mutation of lysine 16, was of interest because it suggested that the site necessary for mating-type expression could be localized near amino acid 16. To further test this genetic mapping, we made an allele (*hhf1-17*) that converted the histidine at position 18 to a tyrosine. When assayed by plasmid shuffle, *hhf1-17* was capable of efficiently replacing the wild-type H4 gene. Although not as defective as the complete NH_2-terminal deletion or *hhf1-10*, the glutamine substitution mutant, the mating frequency for *hhf1-17* was only about 1 to 4%, 1/10 to 1/50 of wild-type levels. Thus, histone H4 contains a site required for normal mating-type expression that spans at least Lys^{16} to His^{18}. In addition, the mechanism of this mating-type gene regulation must not be mediated exclusively through lysine acetylation and deacetylation (*15*). Since *hhf1-17* and *hhf1-21* did not confer temperature-sensitive growth, this phenotype and mating-type gene expression are independent functions affected by different structural features of the H4 NH_2-terminal region.

An analysis (*16*) of isogenic strains that differed only in the H4 allele carried on the plasmid revealed three classes of cell cycle phenotypes as shown in Table 1. Transformants in the first class had wild-type cell cycle patterns and included strains with the *hhf1-13* (Arg^5) and *hhf1-17* (Tyr^{18}) alleles. The second class was distinguished by an increase in the length of the S period and included the strains dependent on *hhf1-14* (Arg^5,Arg^8), *hhf1-15* (Arg^5,Arg^{12}), and *hhf1-21* (Arg^{16}); the lengths of the G_1 and G_2/M

Table 1. Point mutations in the NH_2-terminal domain of histone H4 can cause defective mating ability and slower cell cycle progression through the S and G_2/M periods. The indicated H4 alleles refer to the normal chromosomal copies for hosts MSY155, MSY157, and MSY159, and to the plasmid copy for host MX4-22A (*11*). Isogenic MX4-22A strains were constructed by plasmid shuffle and differed only in the histone H4 mutations that each carried (Fig. 1B). For quantitative mating assays, strains were grown to early exponential phase and about 5×10^7 cells were mixed with an equal number of cells of tester strain MSY108 (*MATα his1*), plated on YPD medium (*21*), and incubated at 28°C for 4 hours. Mating mixtures were then washed from the plates and serial dilutions were spread on selective plates to score the number of diploids and the numbers of each haploid parent. Mating efficiencies were calculated as the percentage of haploid cells that mated to form diploid colonies. The reported values are the average of the number (*n*) of independent measurements listed in the adjacent column. For cell cycle analysis, generation times were calculated from cell counts of cultures during early exponential growth at 28°C in YPD medium. The percentages of cells in each period of the division cycle were determined from flow cytometry analysis. The lengths of the cell cycle periods were then calculated from these percentages as described previously (*8, 22*). The reported values are the average of the number (*n*) of independent measurements listed in the last column of the table.

Host	H4 alleles	Mutation*	Mating efficiency (%)	*n*	Generation time (min)	Cell cycle (min)			*n*
						G_1	S	G_2	
MSY159	*HHF1 HHF2*	WT	53.1	2					
MSY155	*HHF2*	WT	50.6	1					
MSY157	*HHF1*	WT	58.0	1					
MX4-22A	*HHF1*	WT	52.8	6	117	24	31	62	4
MX4-22A	*hhf1-13*	KR^5	53.9	2	116	22	35	59	2
MX4-22A	*hhf1-17*	HY^{18}	3.0	2	114	17	31	66	2
MX4-22A	*hhf1-14*	KR^5,KR^8	55.5	2	125	19	44	62	2
MX4-22A	*hhf1-15*	KR^5,KR^{12}	45.5	2	130	20	45	65	3
MX4-22A	*hhf1-21*	KR^{16}	27.2	3	120	24	41	55	2
MX4-22A	*hhf1-10*	KQ^5,KQ^8, KQ^{12},KQ^{16}	0.006	2	211	14	47	147	6
MX4-22A	*hhf1-8*	$\Delta(G^2\text{-}I^{26})$,$QE^{27}$	0.001	3					

*Wild-type genes are designated "WT." Substitution mutations are designated with single letter amino acid codes: first the wild type, then the substitution, followed by the residue number. Deletions are designated by the first and last amino acids deleted. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

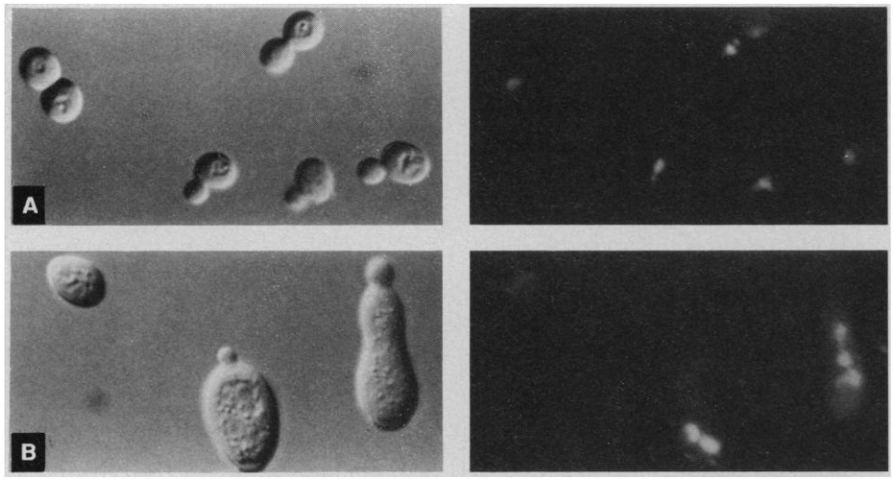

Fig. 2. Strains dependent on *hhf1-10* produce cells with abnormal cellular and nuclear morphologies. Pairs of photomicrographs are shown for cells from isogenic strains that carry either the wild-type H4 gene or *hhf1-10*. Cells from early log-phase cultures were collected and stained with DAPI, a DNA-binding fluorescent dye (*13*). The left of each pair shows the differential interference contrast image of the cells, while the DAPI fluorescence is shown on the right. (**A**) Cells carrying the plasmid with the wild-type histone H4 gene. (**B**) Cells carrying the plasmid with the glutamine substitution allele *hhf1-10*.

periods in these cells were similar to those of the wild type. The glutamine substitution mutant *hhf1-10* formed a third class, and its cells had an S period approximately 50% longer than wild type and a G_2/M period more than twice as long as wild type. It is likely that the aberrant nuclear morphologies of *hhf1-10* cells result from this defective G_2/M function. Thus, one or more of the lysines at positions 8, 12, and 16 must be functionally important during the S and G_2/M periods of the division cycle.

These experiments suggest that the H4 NH_2-terminal domain is structurally and functionally complex. The function that regulates mating-type expression maps to a site encompassing amino acids 16 to 18 and requires features in addition to lysine modification for activity. It is likely that the functions of these site-specific structures are mediated by protein-protein interactions, and, in the case of silent mating-type gene repression, the four *SIR* gene proteins are attractive candidates for such components (*17*).

The mutations that confer S and G_2/M cell-cycle phenotypes define a second region that involves lysine residues 8, 12, and 16. Reversible lysine acetylation could participate in the functions of this region by at least two distinct mechanisms. In the first, the modifying acetyl groups would provide direct structural signals required for function (*18*). In this model, arginine and glutamine substitution mutations should be genetically equivalent since neither is acetylated. The longer S phase seen with both the arginine substitution alleles and *hhf1-10* is consistent with this model, however, the prolonged G_2/M phenotype specific to *hhf1-10* is more difficult to explain.

Alternatively, acetylation would act directly or indirectly by reducing the positive charge at the lysine ϵ-amino groups. Arginine substitutions would affect events requiring acetylation, whereas glutamine substitutions would affect events requiring deacetylation. Since S phase was prolonged by both types of mutations, this predicts that chromosome replication requires dynamic changes in the acetylation state of the NH_2-terminal domain. In contrast, the G_2/M phenotype of *hhf1-10* predicts that histone H4 deacetylation is the dominant requirement for the maturation or segregation of replicated chromosomes. This interpretation is supported by experiments with either sodium butyrate to block deacetylation or antibodies specific for acetylated isoforms of histone H4 (*19*).

The phenotype of a mutant might not result from a defect in H4 function, but from an increase in proteolysis of the altered protein and the assembly of histone-deficient chromatin (*20*). Increased H4 proteolysis is unlikely to account for all the phenotypes observed, however, since the number of different phenotypes and their combinations are difficult to accommodate by a single explanation. Further biochemical, mutational, and genetic suppression studies should begin to provide insights into the mechanisms by which nuclear histones function in chromosome dynamics.

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Δ(*HHT1-HHF1*); MSY157: *MATa ura3-52 lys2-801 ade2-101* Δ(*HHT2-HHF2*); MSY159: *MATa ura3-52 lys2-801 ade2-101*; MX4-22A: *MATa ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) (requires a plasmid with H3 and H4 genes). The construction of strains MSY155, MSY157, and MSY159 has been described (*8*).
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Secondary Structure Is the Major Determinant for Interaction of HIV *rev* Protein with RNA

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A region in the human immunodeficiency virus (HIV) *env* message, with the potential to form a complex secondary structure (designated RRE), interacts with the *rev* protein (Rev). This interaction is believed to mediate export of HIV structural messenger RNAs from the nucleus to the cytoplasm. In this report the regions essential for Rev interaction with the RRE are further characterized and the functional significance of Rev-RRE interaction in vivo is examined. A single hairpin loop structure within the RRE was found to be a primary determinant for Rev binding in vitro and Rev response in vivo. Maintenance of secondary structure, rather than primary nucleotide sequence alone, appeared to be necessary for Rev-RNA interaction, which distinguishes it from the mechanism for cis-acting elements in DNA. Limited changes within the 200 nucleotides, which preserved the proper RRE conformational structure, were well tolerated for Rev binding and function. Thus, variation among the RRE elements present in the diverse HIV isolates would have little, if any, effect on Rev responsiveness.

THE GENOME OF HIV IS MUCH more complex than other retroviruses. In addition to the *gag*, *pol*, and *env* genes present in all retroviruses, it encodes at least seven regulatory proteins. Two of these proteins, encoded by *tat* (*1*) and *rev* (*2*) (referred to as Tat and Rev, respectively) are absolutely required for virus replication (*3*) and, thus, represent attractive targets for therapeutic intervention. The mechanisms proposed for Tat function are diverse, with both transcriptional (*4, 5*) and posttranscriptional components being considered (*5, 6*). In contrast, there is general agreement that Rev exerts its effect at the posttranscriptional level to facilitate export of structural mRNAs, entrapped in the nucleus, to the cytoplasm (*7, 8*). Export is most likely mediated through a cis-acting element, referred to as RRE [originally referred to as CAR (*9*)] (*8*), which is present in *env*.

We and others have recently shown that Rev interacts specifically with RNA that contains the RRE sequence (*10, 11*). The predicted structure formed by base-pair interactions within the RNA that comprise the RRE element is shown in Fig. 1. Solution mapping of the RRE RNA indicates that the structure depicted is likely to be correct (*12*). Through RNA annealing experiments, we have shown that the large base-paired "stem structure" does indeed form and that its formation is required for Rev binding (*10*). This finding could be interpreted in several ways: (i) base-pairing interactions in this region are required for the appropriate formation of the other hairpin structures, one or a combination of which may comprise the site for Rev interaction; (ii) Rev interacts directly with the stem structure itself; or (iii) Rev interacts with the large stem structure and other elements of secondary structure simultaneously. To distinguish between these possibilities, we introduced nucleotide changes into the stem

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Table 1. Activity of the RRE mutations in a Rev-response assay. The mutations shown were cloned into the Cla I–Xba I site of plasmid pIIIAR depicted in Fig. 4 and CHOZip-tat_{III} cells were transfected with 100 ng of each plasmid and 100 ng of the Rev expression vector pSVRev. CAT assays were run for 30 mins, a time found to give a linear response. The percent conversion of [^{14}C]chloramphenicol to acetylated products was determined by liquid scintillation counting of the spots cut from the thin-layer chromatography plate. The percent conversion per minute is given. The relative activity of each mutant is compared to that of pIIIAR, which was assigned a value of 1.0. The low to barely detectable level of CAT activity obtained in the absence of Rev did not allow for quantitation. Rev binding was assessed by visual inspection of the gel retardation autoradiograms obtained from at least three independent experiments. The scoring ranges from best binding (+++) to no binding observed (−).

Plasmid	Conversion (%/min)	Activity	Rev/RRE interaction
pIIIAR	1.46	1.00	+++
HS-1	0.59	0.41	++
HS-2	0.37	0.26	++
HS-1,2	1.13	0.80	+++
HB-1	1.96	1.34	+++
HB-2	1.45	1.00	+++
HSL-6	0.73	0.50	++
HSL-7	0.08	0.05	−
HSL-8L	0.10	0.07	−
HSL-8R	0.08	0.06	−
HSL-8RL	1.83	1.25	++
HSL-9	1.63	1.10	+++
HSL-1	1.81	1.23	+++
HSL-2	1.16	0.80	+++
HSL-3	0.26	0.20	+
HSL-4	1.23	0.80	++
HSL-5	0.07	0.05	−