quency, these phenotypes need not solely result from the activities of the transfected genes.

It is possible that the *myc* oncogene plays a role in this insertion through its action on the control of DNA replication (*22*). If H-*ras* transformation of primary cells results not only from expression of *ras* but also from events at an integration site, it is tempting to speculate that the ready transformation of NIH 3T3 cells by H-*ras* results from their already having undergone the chromosomal events that act to regulate the expression of the transformed *ras* phenotype.


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# Assembly of Functional U1 and U2 Human-Amphibian Hybrid snRNPs in *Xenopus laevis* Oocytes


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**Oligonucleotides complementary to regions of U1 and U2 small nuclear RNAs (snRNAs), when injected into *Xenopus laevis* oocytes, rapidly induced the specific degradation of U1 and U2 snRNAs, respectively, and then themselves were degraded. After such treatment, splicing of simian virus 40 (SV40) late pre-mRNA transcribed from microinjected viral DNA was blocked in oocytes. If before introduction of SV40 DNA into oocytes HeLa cell U1 or U2 snRNAs were injected and allowed to assemble into small nuclear ribonucleoprotein particle (snRNP)–like complexes, SV40 late RNA was as efficiently spliced as in oocytes that did not receive U1 or U2 oligonucleotides. This demonstrates that oocytes can form fully functional hybrid U1 and U2 snRNPs consisting of human snRNA and amphibian proteins.**


IT IS NOW WELL ESTABLISHED THAT several members of the class of snRNPs are required for the splicing of pre-mRNA in higher eukaryotes (*1*). These particles each consist of small uridine-rich RNAs associated with several proteins (*2*). The two most abundant of these, the U1 and U2 snRNPs, are thought to mediate splicing through their interactions with the sequences in pre-mRNA introns at the 5′ splice site (*3–5*) and lariat branchpoint regions (*5, 6*), respectively. Both the proteins and the different U snRNAs in snRNPs are extraordinarily highly conserved in species ranging from lower eukaryotes to humans (*2, 7–10*). Consistent with this is the observation that introduction of U snRNAs from a wide variety of phylogenetic sources into the cytoplasm of *Xenopus laevis* oocytes results in the formation of complexes with the amphibian snRNA binding proteins stockpiled in the cytoplasm (*8–10*). These resemble snRNPs in their antigenicity, nuclear location, and sedimentation coefficient. However, it has not been demonstrated


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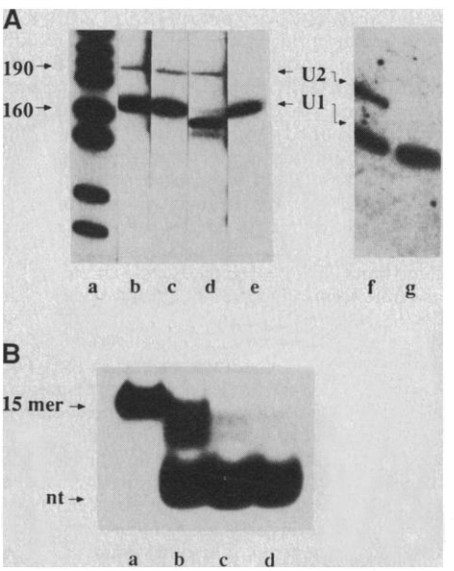


**Fig. 1.** (**A**) Degradation of U1 and U2 snRNA in oocytes injected with U1a and U2b oligos. Left panel: groups of ten oocytes were injected intranuclearly with $H_2O$ (lane b), 20 ng of C oligo (lane c), 5 ng of U1a (lane d), or 20 ng of U2b (lane e). Right panel: RNA from a second batch of oocytes injected with 20 ng of either C oligo (lane f) or U2b (lane g) in which the autoradiogram was exposed for a time interval three times as long as for left panel. The oligonucleotides were delivered into oocyte nuclei in a solution containing 10 m*M* tris-Cl (*p*H 7.5) and 0.1 m*M* EDTA. The sequence of each oligonucleotide is as follows: C oligo, 5′-TCCGGTACCACGACG-3′; U1a, 5′-CTCCCCTGCCAGGTAAGTAT-3′; and U2b, 5′-CAGATACTACACTTG-3′. After being injected, in these and in subsequent experiments, oocytes were incubated at 19°C in modified Barth's solution (*19*). Four hours after injection, RNA was extracted and purified from oocytes as described (*16*). Quantities of RNA equivalent to one oocyte were fractionated on 8% polyacrylamide-urea gels and transferred to a GeneScreenPlus membrane (*20*), and the blot was then hybridized to plasmids containing human U1 or U2 DNA (*21*) that were uniformly labeled with $^{32}P$. It should be noted that the intensity of U1 and U2 snRNAs shown does not reflect the actual ratio of these RNAs in oocytes, since the hybridization was performed sequentially, first with the U2 and then with the U1 probe. The residual intact U1 snRNA detected after injection of U1a was not decreased by injecting larger quantities of this oligonucleotide (*12*). The DNA size markers (lane a) were $^{32}P$-labeled Msp I pBR322 DNA fragments. (**B**) Stability of injected oligonucleotides in oocytes. Oocytes containing 20 ng of U2b oligo 5′ end-labeled with $^{32}P$ were extracted as in (A). The extracts were then analyzed by electrophoresis on 10% polyacrylamide gels. Lane a at 0 min, lane b at 30 min, lane c at 2 hours, and lane d at 4 hours. Labels: 15 mer, a 15-nucleotide oligomer. The nt refers to the position of labeled mononucleotide, which was identified because it migrated slightly more rapidly than $\gamma$-$^{32}P$ but more slowly than inorganic phosphate.

whether such hybrid complexes are functional in the splicing of pre-mRNA.

To test whether human U1 or U2 snRNAs and amphibian proteins can assemble into functional snRNPs, we first had to inactivate the endogenous amphibian U1 and U2 snRNPs. Previous studies have shown that addition of oligonucleotides complementary to the 5′ end of U1 or the internal region of U2 snRNA abolishes splicing of eukaryotic precursor mRNAs in nuclear extracts (*4*, *5*). In order to examine the degradation of snRNAs within an intact cell, oligonucleotides that are complementary either to the twenty 5′-terminal nucleotides of U1 snRNA (U1a oligo) or to nucleotides 28 to 42 within the predicted loop structure of U2 snRNA (U2b oligo) were separately injected into the nuclei of *X. laevis* oocytes. A third oligonucleotide that has no known homology or complementarity to any snRNA was injected as a control (C oligo). RNA blot analysis of oocyte snRNAs, probed with human U1 or U2 DNA, showed that the C oligo caused no detectable degradation of either U1 or U2 snRNAs (Fig. 1A). Injection of the U1a oligo led to the appearance of major and minor U1 snRNA species that were shorter than intact U1 snRNA by approximately 7 and 11 nucleotides, respectively (Fig. 1A, lane d). The U1a oligo failed to induce degradation of the full 20 nucleotides at the 5′ terminus of the U1 snRNA, which is consistent with previously published observations (*11*). By contrast, the U2b oligo induced the disappearance of all detectable oocyte U2 snRNA (Fig. 1A, lanes e and g). The destruction of U2 may be due to destabilization of the U2 particle resulting from disruption of the U2 loop region, such as has been shown to occur in vitro (*5*). The degradation of each snRNA is presumably accomplished by an endogenous ribonuclease (RNase) H activity.

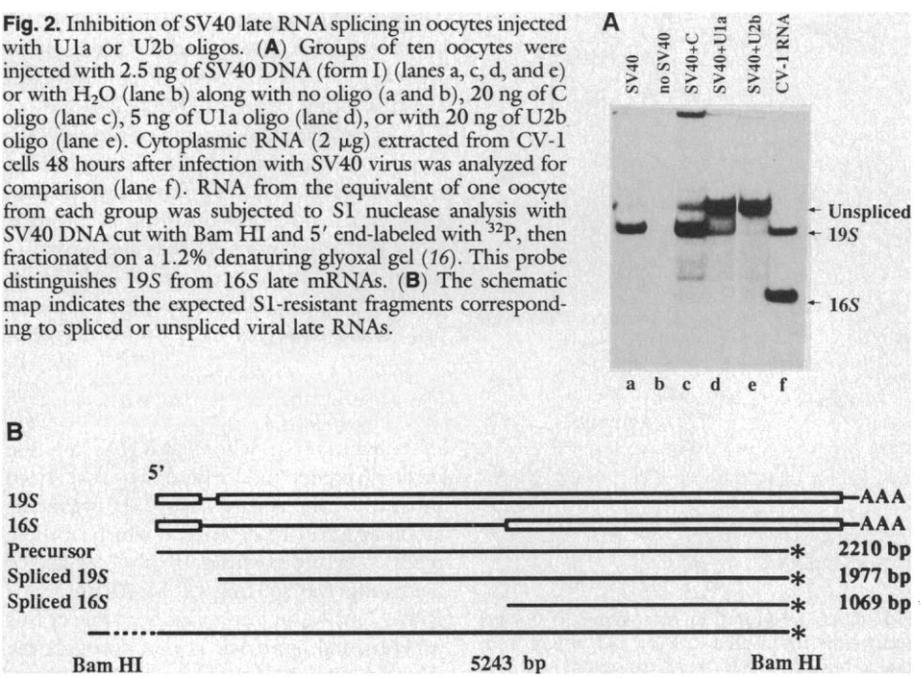


**Fig. 2.** Inhibition of SV40 late RNA splicing in oocytes injected with U1a or U2b oligos. (**A**) Groups of ten oocytes were injected with 2.5 ng of SV40 DNA (form I) (lanes a, c, d, and e) or with $H_2O$ (lane b) along with no oligo (a and b), 20 ng of C oligo (lane c), 5 ng of U1a oligo (lane d), or with 20 ng of U2b oligo (lane e). Cytoplasmic RNA (2 μg) extracted from CV-1 cells 48 hours after infection with SV40 virus was analyzed for comparison (lane f). RNA from the equivalent of one oocyte from each group was subjected to S1 nuclease analysis with SV40 DNA cut with Bam HI and 5′ end-labeled with $^{32}P$, then fractionated on a 1.2% denaturing glyoxal gel (*16*). This probe distinguishes 19*S* from 16*S* late mRNAs. (**B**) The schematic map indicates the expected S1-resistant fragments corresponding to spliced or unspliced viral late RNAs.

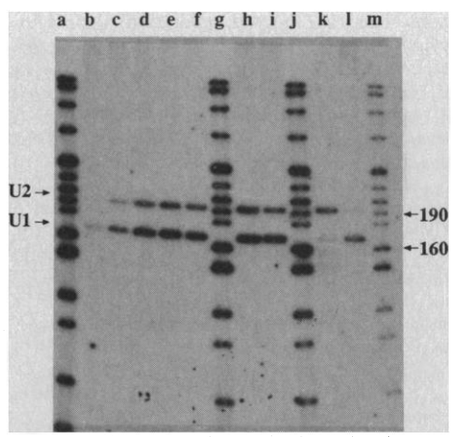


**Fig. 3.** Assembly of U1 and U2 human-amphibian hybrid snRNPs in oocytes. To determine the time course of formation of hybrid snRNPs, we prepared $^{32}P$-labeled HeLa nuclear RNA (30 ng) as reported (*22*) and injected it into the cytoplasm of oocytes, which were then incubated in groups of ten, for 0 min (lane b), 15 min (lane c), 3 hours (lane d), 6 hours (lane e), or 18 hours (lane f). After extraction according to previously published procedures (*23*), an amount of each extract corresponding to 2.5 oocytes was immunoprecipitated with 10 μl of human autoimmune anti-Sm antibodies as described (*15*). RNA purified from the immunoprecipitates was analyzed on 8% polyacrylamide-urea gels. To determine the formation of hybrid snRNPs in U1 or U2 snRNA-inactivated oocytes, we injected similar amounts of HeLa cell nuclear RNA into the cytoplasm of oocytes that had been injected intranuclearly 4 hours before with either 5 ng of U1a (lane h) or 20 ng of U2b oligo (lane i). To determine the sensitivity of the newly formed snRNPs to either U1a or U2b, we introduced HeLa RNA 18 hours before intranuclear injection of either 5 ng of U1a (lane k) or 20 ng of U2b (lane l). After incubation for an additional 18 hours, oocytes were also similarly extracted and immunoprecipitated. Lanes a, g, j, and m contain DNA size markers as indicated in Fig. 1A. As can be seen in lane k, degradation products of HeLa U1 snRNA are faintly visible, suggesting that the partially degraded U1 snRNA is still in a complex that can be recognized by anti-Sm antibodies. The trace amount of snRNA in immunoprecipitates after incubation of oocytes for 0 min in lane b is probably not due to assembly because a direct immunoprecipitation of HeLa cell nuclear RNA, with the use of the same batch of anti-Sm antibodies, yielded comparably low levels of RNA (*12*).

A requirement for testing the function of newly formed snRNP particles was to determine that the injected oligonucleotides were themselves rapidly degraded. The stability of the microinjected oligonucleotides was analyzed by extraction of oocytes at various times after the injection of $^{32}P$ end-labeled U2b oligo (Fig. 1B). Within 2 hours, less than 0.1% of the intact oligonucleotide was detected, during which time the vast majority was converted to nucleotides. Similar observations were made with the U1a oligo (*12*). This is consistent with published observations that indicate that oligonucleotides microinjected into *X. laevis* oocytes have half-lives of ~10 min (*13*).

*Xenopus laevis* oocytes are capable of efficiently splicing simian virus 40 (SV40) late region–specific RNA transcribed from injected viral circular DNA (*14–16*). Far more processed RNA containing the 19*S* exon than that containing the 16*S* exon accumulates in oocytes, while the reverse is true in infected monkey cells. The effects of injected oligonucleotides on the splicing of late SV40 precursor RNA were determined by S1 mapping (Fig. 2). Injection of the C oligo resulted in quantities of spliced RNA that were similar to those in oocytes injected with SV40 DNA alone. By contrast, injection of either U1a or U2b oligos resulted both in a dramatic reduction in the quantities of spliced 19*S* RNA and in the accumulation of unspliced viral late RNA. This result, confirming and extending earlier results (*15*), demonstrates that SV40 late RNA splicing in oocytes requires intact U1 and U2 snRNPs.

The U1 and U2 snRNA-specific oligonucleotides destroyed the function of oocyte snRNAs and then themselves were degraded in a relatively short time. This provided the unique opportunity of determining whether newly assembled snRNP particles generated after injection of nuclear RNA from a differ-

ent species (human HeLa cells) (*8*) are functional. HeLa cell nuclear RNA was injected into the cytoplasm of oocytes. At various times thereafter, extracts of oocytes were prepared and immunoprecipitated with antibodies previously shown to bind a highly conserved antigen (Sm) found in all snRNP particles so far analyzed (*8–10*). RNA was purified from the immunoprecipitates and resolved by gel electrophoresis (Fig. 3). The immunoprecipitable particles that formed reached plateau levels within 3 hours after injection. The U4, U5, and U6 snRNPs were not detected in this experiment presumably because these snRNAs are not abundant in HeLa cells (*8*). Quantities of either U1 or U2 snRNAs immunoprecipitated in oocytes that had been injected 4 hours earlier with either U1a or U2b oligos were similar to those formed in oocytes that were not microinjected with any oligonucleotides. This was consistent with the previously determined time course of stability of the oligonucleotides in oocytes. By contrast, if HeLa RNAs were introduced into oocytes and allowed to assemble into snRNPs, and then U1a or U2b oligos were injected, a substantial proportion of the immunoprecipitated HeLa U1 or U2 snRNAs, respectively, were specifically degraded (Fig. 3). This shows that the sensitivity of the majority of the newly formed snRNP particles to oligonucleotide-targeted degradation was similar to that of the endogenous snRNPs.

In order to determine whether the hybrid snRNP particles were capable of mediating pre-mRNA splicing, three sequential injections were performed. First, U1a or U2b oligos were introduced into oocyte nuclei. Second, 4 hours later, HeLa nuclear RNA or tRNA was injected into the cytoplasm. Finally, after 18 hours, SV40 DNA was injected intranuclearly. Twenty-four hours after the final injection, RNA was extracted from the oocytes and analyzed by S1 nuclease mapping (Fig. 4A). Oocytes that had been injected with either U1a or U2b oligonucleotides and then with tRNA before being injected with SV40 DNA were found to contain mostly unspliced viral late RNA. In contrast, oocytes that had received HeLa nuclear RNA as their second injection produced viral RNA that was predominantly spliced. The restoration of splicing in U2 snRNA-depleted oocytes was reproducibly found to be more efficient than in U1 snRNA-inactivated oocytes. The fact that in both cases the spliced RNA was of the 19*S* rather than the 16*S* type, characteristic of the oocyte but in contrast to the ratio observed in infected cells, indicated that HeLa U1 and U2 snRNAs do not alter the efficiency with which different late mRNA splice sites were utilized.

To further determine whether the newly formed heterologous snRNPs were individually functional, we purified HeLa U1 and U2 snRNAs after polyacrylamide gel electrophoresis and injected them separately into oocytes (Fig. 4B). U1 and U2 snRNAs from HeLa cells were each capable of restoring splicing in U1 or U2 snRNA-inactivated oocytes, respectively. Injection of the U1 snRNA was again slightly less efficient than injection of U2 snRNA in restoring splicing. The inactivated (but stable) endogenous U1 snRNPs containing 5′ end–cleaved U1 snRNA may still have been capable of associating with pre-mRNA, as previously shown in vitro (*17*), and thus were partially competing with the newly formed hybrid U1 snRNPs.

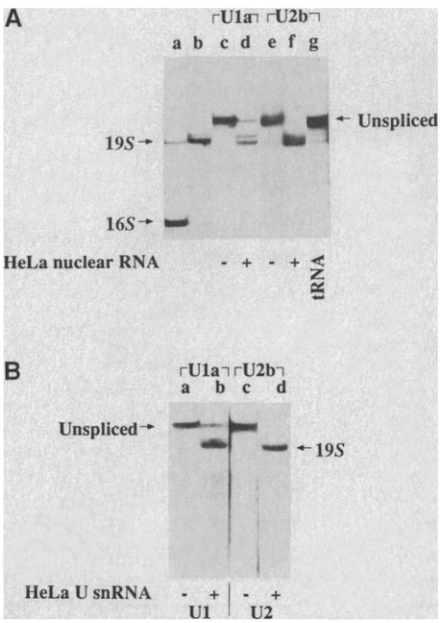


**Fig. 4.** (**A**) Restoration of splicing in oocytes containing inactivated U1 or U2 snRNPs by HeLa nuclear RNA. Five nanograms of U1a oligo (lanes c and d) or 20 ng of U2b oligo (lanes e, f, and g) was injected into oocyte nuclei. Four hours later, the oocytes received in their cytoplasm either no RNA (lanes c and e), 30 ng of HeLa nuclear RNA (lanes d and f), or 100 ng of tRNA (lane g). After an additional 18 hours, each oocyte was injected with 2.5 ng of SV40 DNA and incubated for a further 24 hours. RNA extracted from injected oocytes was subjected to S1 analysis as described in Fig. 2. Lane a, S1-resistant RNA fragments from SV40-infected CV-1 cells; lane b, RNA from oocytes injected with SV40 DNA only. (**B**) Purified HeLa U1 and U2 snRNAs each restore splicing in U1- and U2-inactivated oocytes. Endogenous U1 or U2 snRNAs were inactivated by intranuclear injection of either 5 ng of U1a (lanes a and b) or 20 ng of U2b (lanes c and d). Four hours later HeLa U1 (lane b) or U2 (lane d) snRNAs purified from polyacrylamide-urea gels as described (*24*) in quantities of 0.6 ng (estimated as an approximately sixfold molar excess over the endogenous U1 or U2 snRNAs) were injected, and complexes were allowed to form for 18 hours. SV40 DNA was then injected and 24 hours later RNA was extracted and analyzed by S1 mapping (see legend to Fig. 2). Either HeLa nuclear RNA or purified U1 or U2 snRNA in amounts that were a factor of 3 lower than those indicated failed to restore SV40 late RNA splicing in oocytes injected with either U1a or U2b oligos.

These experiments show that both human U1 and U2 snRNAs are capable of forming functional snRNPs with amphibian proteins. Furthermore, these hybrid particles are capable of interacting with snRNPs formed entirely from amphibian components to form a functional spliceosome. The observation that the 19*S* form of SV40 late mRNA predominates with HeLa U1 and U2 snRNAs suggests that the ratio of 19*S* to 16*S* splice is determined by factors other than U1 and U2 snRNAs. It remains possible that either other snRNAs (U4, U5, or U6) or protein components of the snRNPs might be responsible for the difference in ratio between *X. laevis* oocytes and infected cells. Thus, snRNPs consisting of proteins from HeLa (or monkey) cells and amphibian snRNAs might yield SV40 mRNA in which the predominant species is spliced 16*S* RNA. Oocytes have previously been shown to be capable of assembling snRNP-like particles with snRNAs from a wide variety of organisms, even from *Saccharomyces cerevisiae* (*10*) whose snRNAs are the most divergent in size and sequence from those of higher eukaryotes (*7*). Experiments are in progress to determine which of these types of hybrid particles are also capable of mediating the splicing of SV40 precursor RNA. Finally, an important outcome of this experimental approach is that through the use of in vitro mutagenesis of snRNA genes, the sequences in snRNAs that are required for particle formation and pre-mRNA splicing can be identified. We have recently demonstrated that microinjected plasmids encoding either human or *X. laevis* U2 snRNA are also capable of restoring SV40 RNA splicing in oocytes depleted of their endogenous U2 snRNA (*18*) and thus can initiate such experiments.

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# The Yeast Cell Cycle Gene *CDC34* Encodes a Ubiquitin-Conjugating Enzyme

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**Mutants in the gene *CDC34* of the yeast *Saccharomyces cerevisiae* are defective in the transition from $G_1$ to the S phase of the cell cycle. This gene was cloned and shown to encode a 295-residue protein that has substantial sequence similarity to the product of the yeast *RAD6* gene. The *RAD6* gene is required for a variety of cellular functions including DNA repair and was recently shown to encode a ubiquitin-conjugating enzyme. When produced in *Escherichia coli*, the *CDC34* gene product catalyzed the covalent attachment of ubiquitin to histones H2A and H2B in vitro, demonstrating that the CDC34 protein is another distinct member of the family of ubiquitin-conjugating enzymes. The cell cycle function of *CDC34* is thus likely to be mediated by the ubiquitin-conjugating activity of its product.**

THE CRUCIAL TRANSITION FROM $G_1$ to the S phase of the cell cycle has been the subject of detailed physiological and genetic analysis in the yeast *Saccharomyces cerevisiae*. When yeast cells reach a critical size during the $G_1$ phase of the cell cycle, they perform a function known as "start" and become committed to undergoing a cell division (*1, 2*). The subsequent transition to S phase entails the coordinate initiation of several events, which include bud emergence, spindle morphogenesis, and, finally, DNA replication (*1, 3*). Many of the genetically controlled functions required during these early phases of the cell cycle have been identified by characterization of temperature-sensitive (ts) mutations in the cell division cycle (CDC) genes (*1*). Several of the *cdc* mutants fail to execute the start function under nonpermissive conditions (*1, 2*). Molecular analysis of the start *CDC* genes has shown that some of their products regulate pathways of protein phosphorylation (*4*). The start function is also regulated by yeast analogs of the mammalian guanosine 5′-triphosphate (GTP)–binding proteins $G_S$-α (*5*) and RAS (*6*). After start, functions mediated by the *CDC4* and the *CDC34* gene products are also required before the initiation of chromosomal DNA replication can occur (*3, 7, 8*). Under nonpermissive conditions, ts mutants in *CDC4* and *CDC34* develop numerous abnormal (elongated) buds, and the spindle pole body duplicates but fails to undergo the separation required for spindle formation (*7*).

Our molecular analysis of the functions following start was initiated by characterization of the cloned *CDC4* gene. The deduced amino acid sequence of the 779-residue *CDC4* product contains a repeated motif similar to that found within the β subunits of mammalian GTP-binding proteins, such as $G_S$ and transducin (*9*). Another segment of the *CDC4* product is similar to the products of the *CDC36* gene and the mammalian *ets* oncogene (*10*). Here we describe the molecular and functional analysis of another post-start gene, *CDC34*.

Yeast DNA complementing the ts mutation *cdc34*-1 in strain G101 was isolated from a library of *S. cerevisiae* genomic DNA (*11*) constructed in the vector YRp7 (*12*). The putative *CDC34* gene was mapped within the insert [Fig. 1, (*12*)], and found by R-loop analysis to encode a polyadenylated RNA ~1.2 kb in length (*8*). To verify that the cloned DNA sequence contained *CDC34*, we integrated the complementing plasmid into the yeast genome via homologous recombination and mapped the site of integration. Strain G102 :: *CDC34*, which contains a chromosomally integrated copy of plasmid pCDC34-79, was mated to strain G101 (*13*). Tetrads resulting from the sporulation of this diploid demonstrated tight linkage between *cdc34*-1 and the *TRP1* gene present on the integrated plasmid (19/19 parental ditype tetrads). Integration of plasmid pCDC34-79 at the *cdc34* locus indicated that the plasmid contains the *CDC34* gene.

By conventional genetic mapping (Table 1), we located *CDC34* on the right arm of chromosome IV. The map order is *CEN4-trp1-cdc34-mak21-rad55*. Since the restriction map of *CDC34* differs from that of *RRP1*, which also maps in this region, *cdc34* is not allelic to any previously mapped yeast gene.

To determine whether the distinctive multibudded morphology of arrested *cdc34* cells results from the loss of *CDC34* function under nonpermissive conditions, we inserted the yeast *HIS3* gene within the open reading frame of one copy of *CDC34* in two diploid strains (*14*). When these diploids were sporulated and dissected, 60

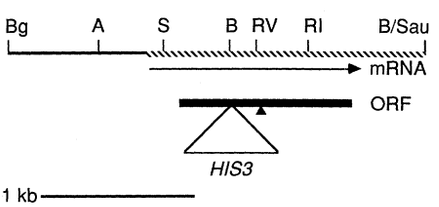


**Fig. 1.** Physical map of the *CDC34* locus. Indicated is part of plasmid pCDC34-79 showing positions of the sequenced yeast genomic DNA insert (striped segment), the *CDC34* mRNA (arrow), the *CDC34* open reading frame (ORF), the insertion used to generate a truncated *cdc34*-3 ORF (*19*) (small triangle), and the site of a *HIS3* insertion used to disrupt *CDC34* (*14*). Designations: A, Apa I; B, Bam HI; Bg, Bgl II; RI, Eco RI; RV, Eco RV; S, Sca I; and B/Sau, Bam HI/Sau 3 AI restriction site junction.

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