Synergistic Activation of Transcription by Bacteriophage λ cI Protein and *E. coli* cAMP Receptor Protein

J. Keith Joung, Deanna M. Koepp, Ann Hochschild*

Two heterologous prokaryotic activators, the bacteriophage λ cI protein (λcI) and the *Escherichia coli* cyclic AMP receptor protein (CRP), were shown to activate transcription synergistically from an artificial promoter bearing binding sites for both proteins. The synergy depends on a functional activation (positive control) surface on each activator. These results imply that both proteins interact directly with RNA polymerase and thus suggest a precise mechanism for transcriptional synergy: the interaction of two activators with two distinct surfaces of RNA polymerase.

The bacteriophage λ cI protein (λcI), which is both a repressor and an activator of transcription (*1*), binds as a dimer to six 17–base pair (bp) operators on the phage chromosome. In a λ lysogen, λcI dimers bind cooperatively to adjacent operators O_R1 and O_R2 in the phage right-operator region (*2*), and the dimer bound at O_R2 contacts RNA polymerase to activate transcription from the adjacent promoter P_{RM} (Fig. 1A) (*3*–*6*). The dimer bound at O_R1 (a high-affinity site) contributes only indirectly to P_{RM} activation by helping another λcI dimer to bind to the weaker-affinity O_R2 site. At high concentrations of λcI, a P_{RM} promoter with only the O_R2 site (centered 42 bp upstream of the transcription start point) can be fully stimulated (*3*). λcI binds DNA with a helix-turn-helix motif (*7*), and residues specifically required for transcriptional activation are located along the solvent-exposed surface of the first helix (*4*, *5*). Evidence suggests that λcI uses this positive control (*pc*) surface to contact the σ subunit of the adjacently bound RNA polymerase (*6*).

The *E. coli* cyclic AMP (cAMP) receptor protein (CRP) is an activator of transcription that regulates the expression of a large number of operons by binding as a dimer to a 22-bp recognition sequence when complexed with cAMP (*8*). CRP consists of a large amino domain that binds cAMP and mediates dimer formation, and a smaller carboxyl domain that binds DNA with a helix-turn-helix motif (*9*, *10*). On binding to its recognition site, CRP induces a sharp bend in the DNA (*10*). Unlike λcI, which can only activate transcription when bound directly upstream of the promoter −35 region, CRP functions when bound at a variety of different distances from the transcription start point (*8*, *11*). At the *lac* promoter, CRP binds to a site centered at position −61.5 relative to the start point of transcription and exerts its stimulatory effect with a solvent-exposed loop in the carboxyl domain that interacts directly with the α subunit of RNA polymerase (*12*). CRP mutants with amino acid substitutions in this activation loop (residues 156 to 164) are defective for positive control of the *lac* promoter, but bind and bend the DNA normally (*13*, *14*).

To determine whether λcI and CRP can function synergistically to activate transcription, we created two artificial promoters derived from a λP_{RM}-*lacZ* fusion (*15*). Both promoters contain a single λcI binding site, O_R2, located at its natural position. In addition, one contains a consensus CRP binding site centered 93.5 bp upstream of the transcription start point (Fig. 1B). These two promoters, $P_{RM}\Delta-50$ and $P_{RM}\Delta-50$/CRP, contain λ sequences that extend as far upstream as position −50 relative to the P_{RM} startpoint.

We performed experiments in the *crp*$^+$ *E. coli* strain MC1000 (*16*). When expressed at a level sufficiently high to ensure occupancy of the O_R2 binding site, λcI stimulated transcription from $P_{RM}\Delta-50$ as measured by both β-galactosidase (β-Gal) and primer extension assays (Fig. 2, A and B). Furthermore, a λcI *pc* mutant defective in the ability to activate wild-type P_{RM} also failed to stimulate our truncated derivative (*17*).

Addition of the CRP binding site upstream of $P_{RM}\Delta-50$ resulted in an increase in *lacZ* transcription in the absence of λcI (Fig. 2, A and B), suggesting that CRP can stimulate the heterologous P_{RM} promoter. In addition, replacement of the high-affinity consensus CRP binding site on $P_{RM}\Delta-50$/CRP with weaker, less canonical CRP binding sites decreased promoter activity (*17*). A CRP *pc* mutant isolated because of its inability to stimulate transcription from the wild-type *lac* promoter (*13*) was also defective in the stimulation of transcription from $P_{RM}\Delta-50$/CRP (*18*). Thus, we conclude that CRP can bind to the consensus site present on the $P_{RM}\Delta-50$/CRP template and stimulate P_{RM} transcription with its previously characterized activation loop.

When the $P_{RM}\Delta-50$/CRP promoter was assayed with both activators, 1420 U of β-Gal activity were produced (Fig. 2A). This is a value much greater than the 364 U expected if the effects of the two activators were simply additive. Primer extension analysis (Fig. 2B) demonstrated that this increase in β-Gal activity was the result of an increase in correctly initiated transcripts from our modified P_{RM} promoter. We conclude that on our artificial template, λcI and CRP activate transcription synergistically.

One mechanism to explain the observed synergy is that each activator uses its positive control surface to contact a distinct surface of RNA polymerase. This hypothesis predicts that replacement of either activator with a *pc* mutant should abolish the synergy. To test this prediction, we assayed our artificial promoters in a Δ*crp* *E. coli* strain with various combinations of wild-type and *pc* mutant forms of λcI and CRP (*19*).

Wild-type λcI stimulated the $P_{RM}\Delta-50$ promoter in the Δ*crp* strain (*20*), whereas the *pc* mutant λcI-E34A (*5*) failed to activate $P_{RM}\Delta-50$ and actually repressed transcription below the basal level (Fig. 3) (*21*). Wild-type CRP stimulated the $P_{RM}\Delta-50$/

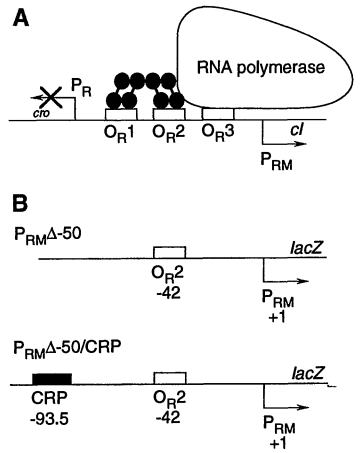

Fig. 1. (**A**) Depiction of bacteriophage λ O_R region in a λ lysogen. The diagram illustrates the three λcI binding sites (open boxes) and associated promoters P_R and P_{RM} (transcription start points indicated with arrows) as well as the configuration of proteins bound to the region. λcI dimers (depicted as dumbbells) bind to O_R1 and O_R2 to repress transcription from P_R. The λcI dimer bound at O_R2 also contacts RNA polymerase to activate transcription from P_{RM}. (**B**) Diagrams of artificial promoters $P_{RM}\Delta-50$ and $P_{RM}\Delta-50$/CRP. Both promoters control expression of a *trp-lacZ* fusion (*32*) and contain an O_R3 site bearing multiple mutations (*31*).

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA.

*To whom correspondence should be addressed.

CRP promoter, whereas the *pc* mutant CRP-H159L did so weakly (Fig. 3). Together, the two wild-type activators worked synergistically: Cells bearing the $P_{RM}\Delta-50$/CRP template and expressing both activators produced 1360 U of β-Gal activity as compared with the 369 U expected if the effects of λcI and CRP were additive (Fig. 3).

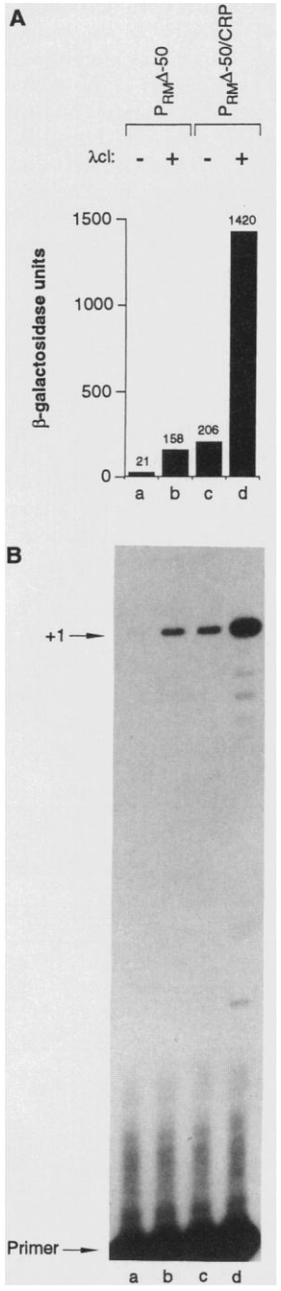

Fig. 2. Effects of λcI and CRP on artificial promoters $P_{RM}\Delta-50$ and $P_{RM}\Delta-50$/CRP. Promoter activities of $P_{RM}\Delta-50$ (lanes a and b) and $P_{RM}\Delta-50$/CRP (lanes c and d) were measured by (**A**) β-Gal assay and (**B**) primer extension assay in *crp*$^+$ strain MC1000 (*16*). Assays were done in strains harboring a control plasmid (lanes a and c) or a λcI-encoding plasmid (lanes b and d). The position of primer extension product produced by correctly initiated P_{RM} transcript is indicated by "+1." Excess, unincorporated primer is indicated.

However, when the CRP *pc* mutant (H159L) (*13*, *22*) and wild-type λcI were expressed in cells bearing the $P_{RM}\Delta-50$/CRP template, the level of β-Gal activity produced was only 293 U (Fig. 3). This level of β-Gal activity is only slightly higher than the 256 U expected if the effects of CRP-H159L and wild-type λcI were additive. λcI stimulated transcription from the single-site ($P_{RM}\Delta-50$) template equally well in the presence of either wild-type CRP or CRP-H159L (Fig. 3), indicating that CRP-H159L did not exert a nonspecific effect on P_{RM} transcription. We therefore conclude that the ability of λcI and CRP to function synergistically depends on the presence of an intact activation loop on CRP.

Cells bearing the $P_{RM}\Delta-50$/CRP template and expressing a λcI *pc* mutant (E34A) and either wild-type CRP or CRP-H159L produced very low levels of β-Gal activity, 83 and 37 U, respectively, indicating that the ability of CRP and λcI to work synergistically also depends on the presence of a functional activation surface on λcI. These values are even lower than those obtained in the presence of either wild-type CRP alone or CRP-H159L alone (266 and 106 U, respectively). This inhibitory effect of the λcI *pc* mutant is consistent with the observation, mentioned above, that λcI-E34A not only fails to stimulate, but actually represses, P_{RM} transcription from the $P_{RM}\Delta-50$ template by a factor of roughly 3.

Although other prokaryotic regulators of transcription have been reported to act synergistically, in these cases only one of the proteins (the target factor) is thought to interact directly with RNA polymerase; the other protein (or support factor contributes only indirectly to activation (*23*). The effects of the activators are synergistic because the support factor (or factors), when bound to its site, either facilitates the binding of the target factor to its DNA site [as is the case, for example, with the λcI dimer bound at O_R1 (see above)] or enhances its ability to interact productively with RNA polymerase (*24*).

Studies with artificial promoters have suggested, however, that two DNA-bound activators might both be capable of interacting directly with RNA polymerase. A pair of CRP dimers can work synergistically to activate transcription from a *lac* promoter derivative bearing two CRP binding sites, one centered at position −61.5 (the natural location of the CRP binding site at the *lac* promoter) and the other at position −93.5 (*25*). Because cooperative DNA binding of the two CRP dimers apparently could not account for the observed synergy, it was suggested that each CRP dimer might interact directly with RNA polymerase.

In another study, S. Busby and colleagues (*26*) demonstrated synergistic activation by a pair of CRP dimers bound to a synthetic promoter bearing CRP binding sites centered at positions −41.5 and −90.5. Using two experimental approaches, they showed that the H159L substitution (see above) eliminates the stimulatory effect of the distally bound dimer, thus providing evidence for a direct contact between the distally bound dimer and RNA polymerase. The synergy was also seen when the anaerobic regulator FNR was bound at the distal position instead of CRP.

The simplest inference from our data is that when bound on our two-site template, λcI contacts the σ subunit of RNA polymerase and CRP contacts the α subunit (Fig. 4). In creating the artificial templates used here, we have not altered the positioning of O_R2 relative to P_{RM}, and as mentioned above, recent evidence suggests that λcI uses its activation surface to contact the σ subunit of the adjacently bound RNA polymerase (6). In the case of CRP, studies with the *lac* promoter indicate that, when bound at position −61.5, CRP uses its activation loop (amino acids 156 to 164) to

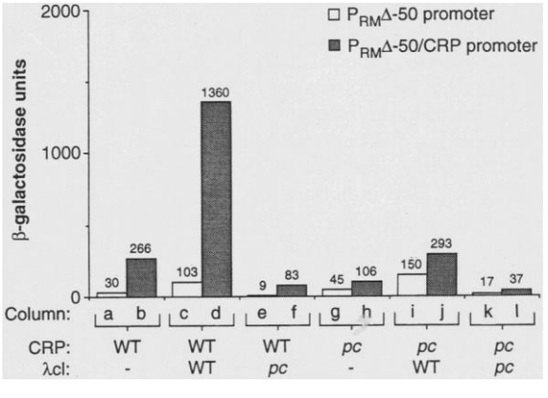

Fig. 3. Effects of wild-type and positive control mutants of λcI and CRP on artificial promoters $P_{RM}\Delta-50$ and $P_{RM}\Delta-50$/CRP. Promoter activities of $P_{RM}\Delta-50$ (columns a, c, e, g, i, and k) and $P_{RM}\Delta-50$/CRP (columns b, d, f, h, j, and l) were measured by β-Gal assay in Δ*crp* strain JCB43Δ*crp39* (*19*). Assays were done in the presence of either wild-type CRP or a CRP *pc* mutant (CRP-H159L) (*22*), with either no λcI, wild-type λcI, or a λcI *pc* mutant (λcI-E34A) (*21*), as indicated.

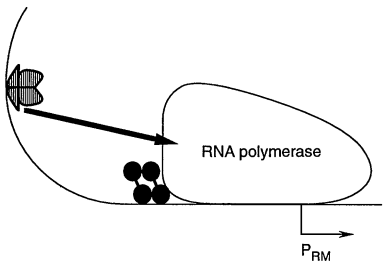

Fig. 4. Model for synergistic activation of our artificial promoter by λcI and CRP. We propose that both the λcI dimer (dumbbells) and the CRP dimer (striped figure) interact directly with RNA polymerase. Possible target sites on RNA polymerase are discussed in the text.

contact the α subunit of RNA polymerase near its COOH-terminal end (*12*). We therefore think it likely that this same target site is contacted by CRP bound at position −93.5 on our artificial template, though we cannot exclude the possibility that when bound at this unusual position CRP uses its activation loop to contact some other target site on the polymerase. A recent demonstration that the α subunit can bind DNA (*27*) prompted the suggestion that the functional consequence of the interaction between CRP and α at the *lac* promoter might be the stabilization of contacts between α and upstream promoter DNA (*27*). On our artificial template, perhaps CRP bound at position −93.5 facilitates the association of α with the DNA in the region spanning the two activator binding sites.

Our report demonstrates that *E. coli* RNA polymerase bound at a single promoter can respond directly to at least two different activators, which in this case likely contact its σ and α subunits. In being able to respond simultaneously to more than one regulator, the bacterial RNA polymerase resembles the basal transcription apparatus in eukaryotes, which is thought to respond to multiple activators at a typical complex promoter (*28*). Our findings with an artificial promoter in *E. coli* raise the possibility that bacterial RNA polymerases might interact with more than one transcriptional activator at some natural promoters (*29*).

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15. Promoters $P_{RM}\Delta-50$ and $P_{RM}\Delta-50$/CRP were derived from the modified P_{RM} promoter on plasmid $EM8O_R5.5$ (*30*). This P_{RM} derivative contains an O_R3 site that has been inactivated by four base-pair alterations (*31*), a G to A substitution at the most promoter-distal base of O_R2 (position −51 relative to the P_{RM} transcription start point), and polylinker DNA sequence separating O_R1 from O_R2. A 168-bp Bam HI–Hind III fragment from plasmid $EM8O_R5.5$ containing the modified P_{RM} promoter without the upstream O_R1 site was ligated to the Bgl II–Hind III vector sequences of two plasmids, p8cons32 and p8lac32. These two vector sequences differ only by the presence of a 34-bp insert in p8cons32 that contains a consensus CRP site (sequence, 5′-AAATGTGATCTAGATCACATTT-3′) positioned just adjacent to the Bgl II site. The resulting plasmids, p8cons/P_{RM} and p8P_{RM}, contain a P_{RM} promoter with and without a consensus CRP site, respectively, centered 77.5 bp upstream of the transcription start point. A 12-bp Xba I linker (NEB linker #1083) was inserted into a Xma I site (ends made flush with Klenow enzyme) present between the Bam HI–Bgl II fusion point and the P_{RM} promoter on plasmids p8cons/P_{RM} and p8P_{RM}. The resulting plasmids contain the desired $P_{RM}\Delta-50$ and $P_{RM}\Delta-50$/CRP promoters on approximately 193- and 227-bp Eco RI–Hind III fragments, respectively. Each of these promoter-containing Eco RI–Hind III fragments was then inserted between the unique Eco RI and Hind III restriction sites of low–copy number plasmid pRW50 (*32*) to generate plasmids $pP_{RM}\Delta-50$ or $pP_{RM}\Delta-50$/CRP on which each promoter controls expression of a *trp-lacZ* fusion gene.
16. In these experiments (Fig. 2), transformants of the *crp*$^+$ strain MC1000 [*ara*D139, Δ(*ara*, *leu*) 7687, Δ*lac* X74, *gal*U, *gal*K, *str*A] (*33*) harboring either $pP_{RM}\Delta-50$ or $pP_{RM}\Delta-50$/CRP were transformed to carbenicillin resistance with either plasmid pLR1 (*31*) or pLR1Δcl (*31*) encoding either wild-type λcI or no λcI, respectively. Simultaneous β-Gal and primer extension assays were performed twice on cultures of these strains with similar results in each case. Values from one experiment are shown (Fig. 2). β-galactosidase assays alone were performed two additional times, again yielding similar results. β-galactosidase assays were performed as described (*34*), except that cultures were grown in LB supplemented with carbenicillin (50 μg/ml) and tetracycline (35 μg/ml). Isolation of RNA, primer labeling, and primer extension assays were all done as described (*25*). The primer was a 20-nucleotide oligomer with the sequence 5′-GCTTGGGATAAGCCAAGTTC-3′ and was complementary to a sequence centered approximately 93 bp downstream of the P_{RM} transcription start point.
17. J. K. Joung, D. M. Koepp, A. Hochschild, unpublished data.
18. Strain MC1000 harboring $pP_{RM}\Delta-50$/CRP was transformed with either plasmid pHA7 (*35*) or pHA7-H159L (*25*) encoding wild-type CRP or *pc* mutant CRP-H159L, respectively. Both plasmids are derivatives of plasmid pBR322 and contain the gene for β-lactam antibiotic resistance. The resulting transformants [which also contained plasmid pACYC184 (*36*)] were assayed for β-Gal activity as described in (*16*) except for the addition of chloramphenicol (30 μg/ml) to the medium. Experiments were repeated twice and similar values were obtained. In one experiment, the $P_{RM}\Delta-50$/CRP promoter produced approximately 180 U of β-Gal in the presence of pHA7 and only 48 U in the presence of pHA7-H159L. We assumed that the effects of wild-type CRP expressed from the MC1000 chromosomal *crp* gene were negligible because plasmid pHA7-H159L expresses very high levels of mutant CRP (*35*).
19. The experiments of Fig. 3 were done in the Δ*crp* strain JCB43Δ*crp39*. This strain was constructed by transducing streptomycin-sensitive strain JCB43 (F$^-$, λ$^-$, *lacZ*$^-$) to streptomycin resistance by infection with P1^{vir} phage grown on a Δ*crp* strain harboring the *crp39* deletion (*37*). Streptomycin-resistant colonies were screened for the linked *crp* deletion by testing for resistance to infection by λcI$^-$ phage. Wild-type CRP and CRP-H159L were expressed from plasmids pHA7 (*35*) and pHA7-H159L (*25*), respectively. Plasmids pA3H, pA3H-E34A, and pA3HΔcI were used to express wild-type λcI, λcI-E34A, or no λcI, respectively. All three plasmids are derivatives of plasmid pACYC184 (*36*) and contain the gene for chloramphenicol resistance. Plasmid pA3H is identical to the previously described plasmid pA3HΔcI (*31*) except that it does not contain a Nsi I–Pst I deletion and therefore contains an intact λcI gene. Plasmid pA3H-E34A is identical to plasmid pA3H except for a single base pair mutation that results in the E34A substitution. β-galactosidase assays were done as in (*18*). Assays were performed twice and yielded similar results. Values from one experiment are shown (Fig. 3).
20. In the set of experiments performed in strain JCB43Δ*crp39*, wild-type λcI stimulated the $P_{RM}\Delta-50$ promoter approximately threefold (Fig. 3, columns a and c, or columns g and i), whereas in the experiments performed in strain MC1000, λcI stimulated the $P_{RM}\Delta-50$ promoter approximately eightfold (Fig. 2, columns a and b). In JCB43Δ*crp39*, we expressed λcI from the medium–copy number plasmid pA3H, whereas in MC1000 we provided λcI from the high–copy number plasmid pLR1. We presume that pA3H expresses a lower level of λcI than does pLR1 and that this lower level is insufficient to saturate the O_R2 site on our templates, thus explaining the decreased activation seen in JCB43Δ*crp39*.
21. Mutant λcI-E34A (*5*) failed to activate, and actually repressed, both wild-type P_{RM} and our various P_{RM} derivatives by a factor of approximately 3. We do not believe that the alanine substitution in this mutant is specifically responsible for the inhibitory effect on P_{RM} transcription. Instead, we believe that the binding of wild-type λcI to O_R2 may have a small inhibitory effect on the binding of RNA polymerase that is normally masked by λcI's activation function.
22. CRP-H159L binds and bends the DNA normally but activates the *lac*P1 promoter (CRP site centered at −61.5) weakly, retaining only 17% of the activation function exhibited by wild-type CRP (*13*).
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38. We thank S. Busby and R. Ebright for helpful discussions and M. Ptashne for helpful suggestions on the manuscript. We are also grateful to S. Busby for providing the pRW50 plasmid and to the J. Beckwith lab for providing the JCB43 strain. Finally, we thank V. Podolny for assistance with DNA sequencing and F. Whipple and A. Pis-Lopez for constructing the λcI-E34A mutation. Supported by NIH grant GM44025 (to A.H.) and by the Searle Scholars Program.

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Complementation by SR Proteins of Pre-mRNA Splicing Reactions Depleted of U1 snRNP

John D. Crispino, Benjamin J. Blencowe, Phillip A. Sharp*

Individual small nuclear ribonucleoproteins (snRNPs) U1, U2, and U4/U6 were removed from nuclear extracts of HeLa cells by antisense affinity depletion. Addition of a highly purified preparation of SR proteins fully restored splicing activity in reactions depleted of U1 snRNP but did not reconstitute splicing in reactions depleted of the other snRNPs. Affinity selection experiments revealed that spliceosomes lacking U1 snRNA formed in the U1 snRNP–depleted reactions reconstituted with SR proteins. Thus, high concentrations of SR proteins facilitate the assembly of precursor messenger RNA (pre-mRNA) into a spliceosome in the absence of interactions with U1 snRNP.

Pre-mRNA splicing takes place within a large complex termed the spliceosome, which contains four snRNP particles (U1, U2, U4/U6, and U5) and also many non-snRNP protein factors (*1*). SR proteins belong to a family of non-snRNP splicing factors that are highly conserved from *Drosophila* to primates and contain extensive repeats of the diamino acid sequence serine-arginine (*2*). Proteins of the SR family have been implicated both in constitutive splicing and in the regulation of alternative splicing (*3*). Recent work suggests that SR proteins function during an early step in the commitment of a substrate to splicing (*4*), facilitate the binding of U1 snRNP to the 5′ splice site (*5*), and also bridge interactions between the 5′ splice site and the branch site (*6*). Here, we show that high concentrations of SR proteins circumvent the requirement for U1 snRNP in the pre-mRNA splicing reaction.

Antisense 2′-O-methyl oligoribonucleotides were used to deplete HeLa cell nuclear extracts of either U1, U2, or U4/U6 snRNPs (*7*). These extracts were not active for the splicing of added pre-mRNA, but complemented each other in any pairwise combination (Fig. 1D) (*7*, *8*). Depleted nuclear extracts were assayed for the presence of snRNAs by Northern (RNA) hybridization (Fig. 1A). In the reaction depleted of U1 snRNP, the amount of this snRNA was reduced approximately to a thousandth of the previous amount, whereas the levels of the nontargeted snRNAs were not greatly affected.

Fractions containing SR proteins complemented splicing of a β-globin substrate in reactions depleted of U1 snRNP (Fig. 1D). Preparations of purified SR proteins were derived from nuclear extracts of HeLa cells as described (*2*) (Fig. 1B). A typical SR preparation also contained a small amount of contaminating snRNAs that were degraded by digestion with micrococcal nuclease. The resulting preparations contained no detectable snRNAs when analyzed by Northern hybridization (Fig. 1C).

After digestion with micrococcal nuclease, SR preparations retained the ability to restore splicing to reactions depleted of U1 snRNP (Fig. 1D). This resistance contrasted with the sensitivity of both the nuclear extract and the U2 snRNP–depleted extract, which did not restore splicing to a U1 snRNP–depleted reaction upon nuclease treatment (Fig. 1D). Nuclear extract and U2 snRNP–depleted extract probably complement the absence of U1 snRNP by contributing functional U1 snRNPs; degradation of the U1 snRNA would be expected to render these preparations inactive. This suggests that the activity of the SR preparation is not the result of residual U1 snRNA. The effects of SR proteins were specific, because their addition had little effect on the extent of splicing of mock-depleted reactions and, in addition, did not restore splicing to either U2 snRNP– or U4/U6 snRNP–depleted reactions (*8*). In titration experiments, the concentration of SR proteins required to complement the U1 snRNP–depleted reactions was approximately 10-fold greater than that of the endogenous concentration (*8*).

The activity of SR preparations complementing a U1 snRNP–depleted reaction was dependent on the particular pre-mRNA. The β-globin substrate was the most active under these conditions, whereas an adenovirus pre-mRNA substrate, Ad1, was also spliced (Fig. 2A), but at approximately 10% of the level observed in the mock-depleted reaction. In the case of the Ad1 substrate, addition of SR proteins to the mock-depleted reaction resulted in the activation of a cryptic 5′ splice site that is located 125 nucleotides downstream of the normal splice site (Fig. 2B). Both sites were also active in the U1 snRNP–depleted reaction supplemented with SR proteins. A third substrate, pPIP85A, which is largely based on sequences in the Ad1 pre-mRNA but with many sequence variations, was not active for splicing upon addition of SR proteins to U1 snRNP–depleted reactions (*8*). Thus, there is a sequence specificity for splicing in the absence of U1 snRNP and in the presence of high concentrations of SR proteins.

To investigate further the role of SR proteins in the facilitation of splicing in U1 snRNP–depleted reactions, spliceosomes were formed in reactions containing 0.2 μg of substrate RNA that had been synthesized with biotinylated uridine triphosphate (*9*). This amount of substrate corresponded to at least a 100-fold excess over the minute amounts of U1 snRNA in the depleted reactions. Biotinylated β-globin pre-mRNAs were incubated under splicing conditions for 40 min in mock-depleted, U1 snRNP–depleted, and U2 snRNP–depleted reactions either with or without added SR proteins; the extent of splicing was assayed by addition of a trace amount of labeled substrate (Fig. 3A).

The spliceosomes from these reactions were recovered by chromatography on streptavidin agarose beads; and the bound RNA was analyzed by Northern blot analysis (Fig. 3B). All six snRNAs were associated with the spliceosome in reactions containing mock-depleted extract. The addition of excess SR proteins had a small stimulatory effect on splicing and also on the amount of snRNAs in spliceosomes. Trace amounts of U2, U4, U5, and U6 snRNAs

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*To whom correspondence should be addressed.