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Genetics

The *Ste* locus, a component of the parasitic *cry-Ste* system of *Drosophila melanogaster*, encodes a protein that forms crystals in primary spermatocytes and mimics properties of the β subunit of casein kinase 2

(repetitive DNA/meiosis)

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ABSTRACT **Males of *Drosophila melanogaster* lacking the Y chromosome-linked crystal locus show multiple meiotic alterations, including chromosome disorganization and prominent crystal formation in primary spermatocytes. These alterations are due to the derepression of the X chromosome-linked Stellate sequences. To understand how the derepression of the Stellate elements gives rise to these abnormalities, we have expressed the protein encoded by the Stellate sequences in bacteria and produced an antibody against the fusion protein. Immunostaining of crystal⁻ testes has clearly shown that the Stellate protein is a major component of the crystals. Moreover, *in vitro* experiments have shown that this protein can interact with the catalytic α subunit of casein kinase 2 enzyme, altering its activity.**

The Y chromosome is known to be essential for male fertility in *Drosophila melanogaster* (1). Previous work demonstrated that many aspects of the X0 phenotype reflect an unusual negative regulatory interaction that normally occurs between the X chromosome-linked Stellate (*Ste*) locus and the Y chromosome-linked crystal (*cry*) or Suppressor of Stellate [*Su*(*Ste*)] locus (2–6). That is, the *Ste* and *cry* loci are normally silent. Deficiencies of *cry* lead to the derepression of the *Ste* elements in the male germ line (3, 4, 7) and lead to the mutant phenotype. Males lacking the Y-linked *cry* locus exhibit needle or star-shaped crystalline aggregates in the nuclei and the cytoplasm of primary spermatocytes (2, 8) and several other meiotic defects such as an undercondensation of meiotic chromosomes (9) and an altered distribution of mitochondria, leading, in many cases, to complete sterility. Both the formation and shape of crystals and the strength of the other meiotic abnormalities depend on the allelic state of the X-linked *Ste* locus (2, 6). Males carrying a cry^- Y chromosome and a *Ste* allele show star-shaped crystals and are sterile. cry^- males carrying Ste^+ alleles show needle-shaped crystals and are somewhat fertile, but they evince genetic abnormalities such as nondisjunction and meiotic drive (2, 6). The *Ste* and *cry* loci are composed of homologous repetitive sequences (3, 4). The *cry* locus contains about 80 copies of an 800-bp *Cfo* I fragment (3), and *Ste* contains three classes of repeats giving 950-, 1100-, and 1150-bp *Cfo* I fragments (3, 6). The repeats are present in more than 200 copies in *Ste* alleles, whereas they are an order of magnitude less abundant in Ste^+ (3, 6). By high-resolution fluorescent *in situ* hybridization to larval neuroblast chromosomes, the *cry* locus has been localized to the h11 region (6) of the mitotic prometaphase map of the Y chromosome (10). Two clusters of the *Ste* sequences have also been localized, one to the 12E1-2 euchromatic region of the X-polytene chromosome map, and the other to the h27 region of the mitotic prometaphase heterochromatin map of the X chromosome (6).

The *Ste* sequences contain two introns and are efficiently and correctly transcribed only in testes of cry^- males, producing an abundant 750-bp poly(A)$^+$ transcript that is absent in wild-type males (4). Sequence analysis has revealed that the Ste protein shares extensive homology with the β subunit of casein kinase 2 (CK2) (4, 11, 12), an enzyme that is able to aggregate in filamentous structures (13) and, among its multiple functions (14), is involved in topoisomerase II activity (15), which is essential for chromosome condensation and segregation (16–18).

To further examine the relationship among the Ste protein, CK2, and the cry^- phenotype, we isolated antibodies against a Ste fusion protein. Using this antibody, we show here that the Ste protein is absent in XY males but is a major component of the crystalline aggregates in cry^- males. We have used the bacterially expressed Ste protein in *in vitro* assays to show that it can interact with the α subunit of CK2 to form a complex exhibiting some properties of an active $\alpha_2\beta_2$ holoenzyme.

MATERIALS AND METHODS

Construction of the MS2–Stellate Fusion Protein and Production of a Polyclonal Ste Antibody. Restriction analyses, genetic engineering procedures, and Western blots were performed as described (19). Sequences of the clones of interest were performed using a Sequenase version 2.0 kit (United States Biochemical).

A 17-mer (5′-TGCACGTAGTCGGTGGG-3′) derived from the Stellate sequence was used as primer. Computer analysis was performed using GLORIA and ACNUC packages developed by the Bioinformatic Group from CNR Research Area of BARI (Italy) and resident at the Italian EMBnet node in Tecnopolis (Valenzano-Bari).

To construct the Ste expression vector, we used a clone, named G1 (kindly provided by K. J. Livak, Central Research and Development, E. I. DuPont de Nemours Experimental Station, Wilmington, DE), consisting of an 843-bp *Eco*RI fragment inserted in the pIBI30 vector. To subclone only the protein-producing sequence, a 665-bp *Sac* I–*Eco*RI fragment, obtained by digestion of G1 DNA, was inserted in *Sac* I–*Eco*RI-digested pUC19 (20). The resulting subclone was designated pUSTE6. TG1 $CaCl_2$ competent cells were trans-

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Abbreviation: CK2, casein kinase 2.

formed with standard techniques. To express the Stellate protein, the pEX34 bacterial expression system was chosen. This system allows cloning in all three frames and produces a fusion protein linked to the NH_2-terminal region (about 10,000 Da) of MS2 polymerase (21). DHK12 electrocompetent cells were transformed by electroporation (19). pUSTE6 was digested with *Bam*HI–*Eco*RI, and a 660-bp fragment was obtained, which codes for 171 amino acids of Stellate protein. This fragment was inserted in the pEX34b expression vector digested with *Bam*HI–*Bgl* II (*Eco*RI site of the fragment and *Bgl* II site of the plasmid were previously filled in with the Klenow fragment of DNA polymerase to allow cloning in the right orientation). After cell transformation by electroporation, screening for positive clones was performed using SDS/12% PAGE by searching for the expression of MS2–Stellate fusion protein after temperature induction. The screening for production of fusion protein after induction was performed as described (21). After induction, one of the transformed clones that produced a band of the expected size for a chimeric protein (≈29,000 Da) was identified. This clone was designated pEXS6. The correct reading frame of the fusion protein was checked by sequence analysis using as primer an oligonucleotide hybridizing to the 5′ end of the cloned Stellate fragment. The purification of the fusion protein was performed as described (21). The band corresponding to the fusion protein was cut directly from the gel and used to immunize mice. The antiserum (TS1) raised against MS2–Stellate fusion protein was analyzed by Western blot on pEXS6 extracts.

Immunofluorescence. For indirect immunofluorescence, testis preparations were fixed as described (22). After washing three times in PBS (5 min each), the slides were incubated for 1 hr with 20 μl of TS1 antiserum diluted 1:15 in PBS. They were then washed two times in PBS, incubated for 1 hr with the secondary antibody [fluorescein-labeled sheep anti-mouse immunoglobulin, $F(ab')_2$ fragment; Boehringer Mannheim] diluted 1:20 in PBS, washed thoroughly with PBS, and mounted in PBS. Sequential staining with Hoechst 33258 was performed according to Palumbo *et al.* (6). Fluorescent images with the different stainings were recorded separately by a cooled charge-coupled device camera (Photometrics) as grey-scale digital images and then pseudocolored and merged by the ADOBE PHOTOSHOP program.

***In Vitro* Assay for Binding of the Ste Protein to the CK2 α Subunit.** Recombinant α and β subunits of human protein kinase CK2 were prepared as described (23). Rat liver CK2 was purified to near homogeneity essentially as reported by Meggio *et al.* (24) with a subsequent FPLC Mono Q (Pharmacia) chromatographic step. CK2 phosphorylation activity toward synthetic peptide substrates was monitored essentially as described (25), except for the presence in all experiments of 0.2 M urea in the phosphorylation assay. The phosphorylation of calmodulin and the evaluation of the phosphate incorporated were accomplished as described by Meggio *et al.* (26). For the thermal inactivation assay, isolated CK2 α subunit, either alone or after addition of the Ste fusion protein, was preincubated at 45°C for 0–10 min in 50 mM Tris·HCl (pH 7.5)/0.2 M urea. The samples were immediately ice cooled, and the residual activity was determined as described above. Control experiments were performed by replacing the Ste fusion protein with either CK2 β subunit or bovine serum albumin. For the sucrose density gradient analysis, sucrose density ultracentrifugation of the Ste fusion protein either alone or added in 17-fold molar excess to the α subunit was run in the presence of 0.2 M urea for 15 hr at 37,000 rpm in a Beckman SW39 rotor. Twenty fractions were collected and analyzed on SDS/15% PAGE after precipitation with 20% (wt/vol) trichloroacetic acid in the presence of 2 μg of bovine serum albumin. The immunodetection of the Ste protein on the blotted gels was evaluated with specific Ste antibody.

RESULTS AND DISCUSSION

Immunolocalization of the Ste Protein in Testes. The coding sequence of a Stellate cDNA previously obtained by Livak (4) was subcloned in pEX34 bacterial expression vector, and the fusion protein was used to immunize mice. We obtained a polyclonal antibody, denoted TS1, that reacts with a single band of the expected size of 29,000 Da present exclusively in the induced pEXS6 extracts (Fig. 1*a*). This antibody identifies the Ste protein as a single band on Western blots of protein extracts of only *cry*⁻ males (Fig. 1*b*). The protein recognized by TS1 migrates as a 20,000-Da protein on SDS/PAGE. It is abundant and visible after Coomassie blue staining of testis extracts of *cry*⁻ males, but it is not detected in extracts of normal males (data not shown).

To examine the cellular localization of the Ste protein in the male germ line, the TS1 antibody was used to immunostain primary spermatocytes in *cry*⁻ males. We prepared testis squashes from males with two different sex chromosome constitutions: X, *Salve-1 Ste*⁺/*cry*⁻ Y and X, *301.2 Ste*/*cry*⁻ Y. The *cry*⁻ Y corresponds to the *Df(Y) cry1* that lacks the *cry* locus (6); the X, *Salve-1 Ste*⁺ chromosome carries 52 copies of the *Ste* sequences, while the X, *301.2 Ste* chromosome carries 300 copies (6). Previous studies (6) have shown that these two X chromosomes result in crystals with distinct morphologies as visualized with phase-contrast microscopy. As shown in Fig. 2 *a–d*, the antibody decorates the needle-shaped cytoplasmic crystalline aggregates that are easily visible under phase-contrast microscopy in the spermatocytes of *Salve-1 Ste*⁺/*cry*⁻ males. In addition, we detect a number of small, often star-shaped nuclear crystals located within spermatocyte nuclei (Fig. 2*d*). Immunostaining also reveals that both intranuclear and cytoplasmic crystals first appear in very young spermatocytes; at this stage, a fluorescent spot is visible within each nucleus, which eventually develops into a star-shaped aggregate in mature spermatocytes (Fig. 2 *a* and *b*). In addition, a few needle-shaped crystals are visible (Fig. 2*b*); they are irregularly distributed within the cytoplasm and progressively increase in size in more mature stages. Interestingly, in the cytoplasm of fully grown spermatocytes, a myriad of tiny needle-shaped crystals can be clearly observed (Fig. 2*d*). These are probably responsible for the homogeneous cytoplasmic staining observed in earlier stages. Conspicuous star-shaped crystals can be observed in both the nuclei and the cytoplasm of primary spermatocytes of *301.2 Ste*/*cry*⁻ males (Fig. 2 *e* and *f*). As above, the intranuclear aggregates start to develop from

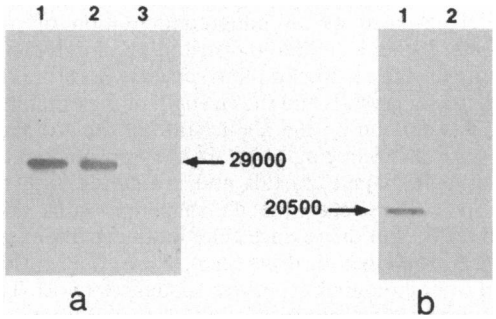

FIG. 1. (*a*) Western blot analysis of bacterial extracts incubated with the TS1 antiserum. Lane 1, purified induced extract of pEXS6; lane 2, induced pEXS6 extract; lane 3, extract of pEXS6 with no induction. It is clear that the TS1 antiserum is specific for the MS2–Stellate fusion protein (29,000 Da) and does not react with the noninduced pEXS6 extracts. (*b*) Western blot of *Drosophila* testis extracts incubated with the TS1 antiserum. Lane 1, extract from *301.2 Ste*/*cry*⁻ males; lane 2, extract from *301.2 Ste*/*cry*⁺ males. A band of 20,000 Da hybridizes with the antiserum only in the lane of the extracts of *301.2Ste*/*cry*⁻ males, indicating that it corresponds to the Ste protein.

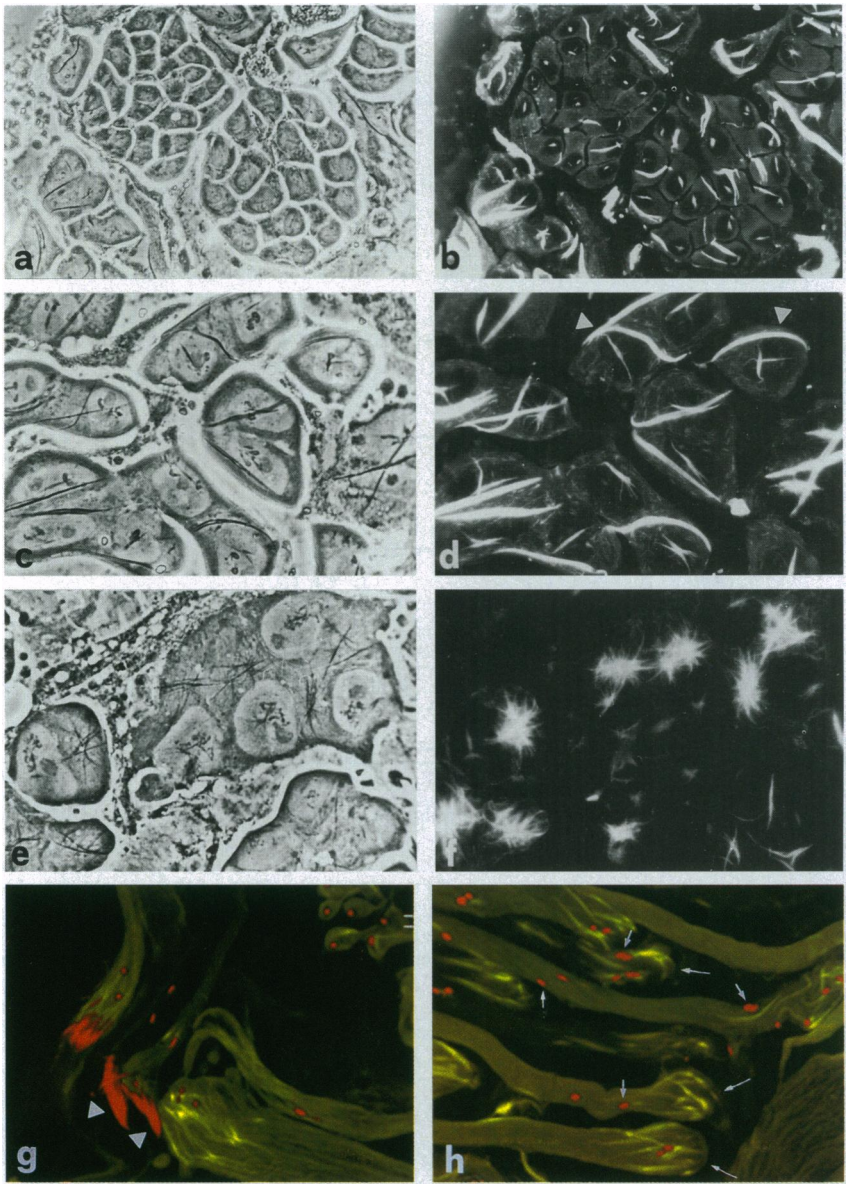

FIG. 2. Immunofluorescence staining of testes from males of different genetic constitutions. Phase contrast (*a* and *c*) and immunofluorescence (*b* and *d*) of *Salve1 Ste*$^+$/*cry*$^-$ are shown. (*a* and *b*) Young spermatocyte cysts showing the development of the star-shaped intranuclear crystals and the conspicuous needle-shaped cytoplasmic crystals. (*c* and *d*) Mature spermatocytes exhibiting fully developed intranuclear star-shaped crystals, huge needle-shaped aggregates (arrowheads), and a myriad of tiny crystals scattered in the cytoplasm. Phase contrast (*e*) and immunofluorescence (*f*) of *301.2 Ste*/*cry*$^-$ are shown. Mature spermatocytes showing fully grown star-shaped aggregates. (*g* and *h*) Sperm bundles of *Salve1 Ste*$^+$/*cry*$^-$. Note that in *g* some bundles show regular heads (arrowheads), whereas in *h* all the bundles are full of crystals (long arrows) with some undercondensed chromatin (short arrows). Images in *g* and *h* were recorded by a charge-coupled device camera, pseudocolored and merged as described in *Materials and Methods.* (Red, chromatin; yellow, crystals; green, tubulin.). (×350.)

small spots within very young spermatocyte nuclei, whereas cytoplasmic crystals, which appear star-shaped from the very beginning of their growth, occupy variable positions among the nuclei and noticeably increase in size in mature spermatocytes. In the final stages of spermatogenesis, sperm bundles full of crystals and containing various amounts of uncondensed chromatin (Fig. 2 *g* and *h*) are observed in *cry*$^-$ males. The frequency of this type of abnormal sperm bundles correlates with the number of *Ste* copies in the two genotypes examined.

These results indicate that the Ste protein constitutes a major component of the crystalline aggregates.

Functional Homology Between the Ste Protein and the CK2 β Subunit. The nondisjunction and chromosome condensation defects observed in *cry*$^-$ males might be the consequence of a mechanical disruption of meiosis and cell division caused by the nuclear and cytoplasmic crystals. In this case, the Ste protein might exert its effect simply by being expressed abundantly enough to form crystalline aggregates. An alternative possibility, based on the Ste homology to the CK2 β subunit (4, 11, 12), is that *Ste* encodes an active component or interrupts the function of CK2. To test this possibility, we assayed the functional homology between the Ste protein and the β subunit of CK2 by using an *in vitro* assay.

Previous studies have shown that the mammalian α subunit, which is the catalytic subunit of CK2, assembles with the β subunit *in vitro* to form $\alpha_2\beta_2$ heterotetramers (23, 27–30). When added in equimolar amounts, the Ste fusion protein fails to interact with the CK2 α subunit (Fig. 3*b*). However, if the concentration of the Ste fusion protein is increased up to a molar excess of at least 10-fold over the α subunit, an increase of basal catalytic activity is observed of the same order of that induced by the β subunit (Fig. 3*b*). No stimulation of CK2

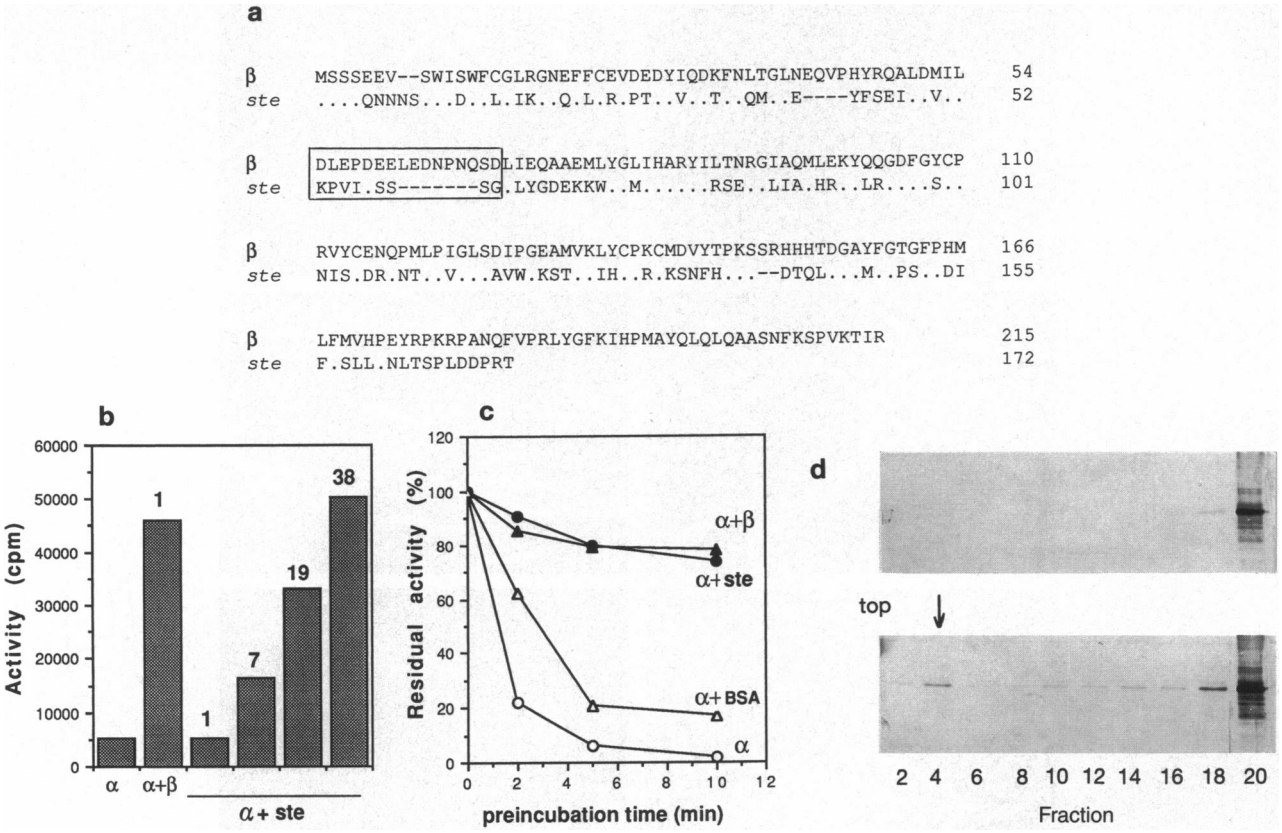

FIG. 3. Biochemical characterization of the Stellate fusion protein in comparison human CK2 β subunit. (*a*) Homology between the human CK2 β subunit and the Ste protein. The acidic domain (residues 55–70) responsible for down-regulation is boxed. (*b*) Stimulation of the catalytic activity of the human CK2 α subunit by the β subunit and the Ste protein. Activity was monitored with the peptide substrate RRRAADSDDDDD (1 mM) in the presence of 0.2 M urea. Numbers above the bars denote the molar ratio relative to the α subunit. (*c*) Thermal inactivation of the human CK2 α subunit: protective effect of the β subunit (equimolar concentration) and the Ste protein (17-fold molar excess). Experimental conditions are detailed under *Materials and Methods.* BSA, bovine serum albumin. (*d*) Sucrose density gradient ultracentrifugation of the Ste fusion protein either alone (*Upper*) or added in a 17-fold molar excess to the human α subunit (*Lower*). Individual fractions were subjected to SDS/15% PAGE analysis as described in *Materials and Methods.* The immunodetection of the Ste protein with specific Ste antibody on the blotted gels is shown. The arrow denotes the position of CK2 activity peak along the gradient.

α-subunit activity is observed by replacing the Ste fusion protein with equivalent concentration of bovine serum albumin (data not shown). Stimulation of catalytic activity is evident only if Ste fusion protein is added to the catalytic α subunit. On the contrary, the Ste fusion protein has no stimulatory but rather a slightly inhibitory effect when it is added to either rat liver CK2 or to reconstituted human CK2 holoenzyme, both exhibiting the heterotetrameric $\alpha_2\beta_2$ structure (data not shown). The lower affinity of the Ste fusion protein for the CK2 α subunit as compared to the β subunit, even if we cannot exclude some effect due to the use of a fusion protein, is probably due to their differences in the C-terminal domain (residues 171–181 of the β subunit) (see Fig. 3*a*), which is known to be important for the interaction with the α subunit (25).

A 17-fold molar excess of Ste fusion protein also prevents thermal inactivation of the α subunit, thus mimicking the effect of equimolar concentrations of the β subunit (Fig. 3*c*).

To gain more direct evidence about the interactions between Ste protein and the CK2 α subunit, sucrose gradient ultracentrifugation experiments were performed. To overcome the insolubility of the Ste fusion protein, ultracentrifugation was performed in the presence of 0.2 M urea, which is compatible with CK2 activity (30). These conditions, however, seem to weaken the interactions between the Ste fusion protein and the CK2 α subunit, thus hindering the clear-cut detection of oligomeric complexes, which were monitored only in two experiments out of three. However, also in the third experiment, a positive interaction of the Ste fusion protein with the CK2 α subunit was revealed by its immunodetection along the gradient. As shown in Fig. 3*d*, the addition of the CK2 α subunit results in significant amounts of the Ste fusion protein displaying a decreased sedimentation coefficient, either comigrating with the CK2 α subunit (fraction 4) or smeared in several fractions in the middle of the gradient (*Lower*). As shown in Table 1, phosphorylation of calmodulin by the reconstituted $\alpha_2\beta_2$ holoenzyme is stimulated 15.8-fold by polylysine. However, almost no effect of polylysine is observed when the α subunit is in the presence of a 17-fold molar excess of Ste fusion protein. As highlighted elsewhere (26), an acidic region of the β subunit, residues 55–64, is responsible for responsiveness to polylysine stimulation, which is especially evident with calmodulin as a substrate. This region is not conserved in the putative Ste product (see Fig. 3*a*).

Conclusions. Our data lead us to propose the following model to explain the action of the Ste protein in spermatocytes of *cry*$^-$ males. The functional homology of the Ste protein with the β subunit of CK2 suggests that the Ste protein may compete with the normal β subunit by binding to the α catalytic subunit. This could give rise to oligomers with altered activity and/or regulatory properties, as indicated by the lack of stimulation by polylysine when the β subunit is replaced by Ste fusion protein (see Table 1). Pertinent to this is the observation that a number of CK2 targets are fully dependent on these polybasic effectors (including several histones; ref. 26) for their phosphorylation (e.g., calmodulin, ornithine decarboxylase, clathrin β light chain, and elongation factor 1). It could be hypothesized therefore that in the presence of high

Table 1. Failure of the Stellate protein to mediate polylysine stimulation of calmodulin phosphorylation by human CK2 α subunit

Enzyme	Calmodulin phosphorylation, cpm		Fold stimulation
	Control	+ polylysine (84 nM)	
$\alpha + \beta$ (1:1)	507	8054	15.8
α + Ste (1:17)	856	1374	1.6

α and β refer to the α and β subunits of CK2.

Ste protein concentration, the activity of CK2 toward these targets would be selectively decreased. This mode of regulation could be especially effective in nuclei due to the nuclear localization of histones, some of which have been shown to surrogate for polylysine as activators of CK2 holoenzyme (26). Topoisomerase II, together with DNA ligase I, is among the nuclear CK2 targets whose activity is triggered by phosphorylation (for review, see ref. 31). Consequently, a decreased activity of topoisomerase II could be a major factor in causing the spectrum of chromosome alterations observed in *cry*$^-$ male germ cells, although involvement of other meiotic proteins regulated by CK2 cannot be excluded.

Two important points remain, however, to be clarified. One is the mechanism by which crystal represses the Stellate sequences. The second question is how the *Ste* loci maintain their coding capacity despite the fact that they are normally inactive and thus probably dispensable. The adventitious nature of these sequences is indicated by the finding that, with the exception of a single copy located in the Y chromosome of *Drosophila simulans*, such sequences are not present in any other member of the *melanogaster* group of *Drosophila* (3). The present data, showing the absence of Ste protein in normal males, further support the suggestion that the *Ste-cry* system is dispensable and, under this hypothesis, identify an evolutionary strategy evolved by a parasitic genetic system to actively maintain itself. It consists in being nested into a meiotic physiological process so intimately that it mimics essential functions. Thus, any perturbation of *cry-Ste* system results in a perturbation of the entire meiotic process, with a negative impact on fitness.

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