hemoprotein reductase activity therefore indicates that all the components necessary for squalene epoxidation are assembled on the hemoglobin molecule. It is not yet clear whether an additional oxidase is required for epoxidation, or whether this hemoglobin carries oxygen for use in the many other oxygen-requiring reactions involved in the conversion of squalene to cholesterol.

A clue to the physiologic role of this unusual molecule is the observation that female *Ascaris* worms have an order of magnitude more hemoglobin in their perienteric hemolymph than do males. Females each produce about 0.5 g of eggs (about 10% of total body weight) per day (*23*). The eggs are rich in sterols for use in the membranes of the developing larvae [sterols comprise approximately 2.4% of the dry weight of *Ascaris* eggs (*24*), comparable to the proportions found in ovine eggs (*25*)]. Thus, the female worms must synthesize large amounts of sterol, by a pathway that requires molecular oxygen, even though they live in the oxygen-poor intestinal folds. The parasites appear to have solved the problem by elaborating a hemoglobin that can sequester any available oxygen and channel it directly into cholesterol biosynthesis. Several related parasitic nematodes also have oxygen-avid hemoglobins (*22*, *26*), which may therefore represent a special adaptation for intestinal helminths, or perhaps a more general feature of microaerophilic eukaryotes.

REFERENCES AND NOTES

1. K. Warren, *The Biology of Parasitism* (Liss, New York, 1988).
2. _____ and A. Mahmoud, *Tropical and Geographical Medicine* (McGraw-Hill, New York, ed. 2, 1989).
3. Z. Pawlowski, *Rev. Infect. Dis.* **4**, 806 (1982).
4. L. Stephenson, *Parasitology* **81**, 221 (1980).
5. D. Crompton, *Trans. R. Soc. Trop. Med. Hyg.* **80**, 697 (1986).
6. C. Bryant, *Adv. Parasitol.* **16**, 3111 (1978).
7. K. S. Cheah, *Biochim. Biophys. Acta* **387**, 107 (1975).
8. H. Davenport, *Proc. R. Soc. London Ser. B* **136**, 255 (1949).
9. K. Hamada, T. Okazaki, R. Shukuya, K. Kaziro, *J. Biochem. (Tokyo)* **52**, 290 (1962).
10. Q. Gibson and M. Smith, *Proc. R. Soc. London Ser. B* **163**, 206 (1965).
11. T. Okazaki, B. A. Wittenberg, R. W. Briehl, J. B. Wittenberg, *Biochim. Biophys. Acta* **140**, 258 (1967).
12. K. Hamada, T. Okazaki, R. Shukuya, K. Kaziro, *J. Biochem. (Tokyo)* **53**, 484 (1963).
13. B. Wittenberg, T. Okazaki, J. Wittenberg, *Biochim. Biophys. Acta* **111**, 485 (1965).
14. S. Darawshe, Y. Tsafadyah, E. Daniel, *Biochem. J.* **242**, 689 (1987).
15. J. Barrett, *Biochemistry of Parasitic Helminths* (University Park Press, Baltimore, 1981).
16. H. Bunn and B. Forget, *Hemoglobin: Molecular, Genetic and Clinical Aspects* (Saunders, Philadelphia, 1986).
17. M. Smith and D. Lee, *Proc. R. Soc. London Ser. B* **157**, 234 (1963).
18. R. E. Weber, *Am. Zool.* **20**, 79 (1980).
19. D. Keilin, *Proc. R. Soc. London Ser. B* **98**, 312 (1925).
20. B. M'Baya and F. Karst, *Biochem. Biophys. Res. Commun.* **147**, 556 (1987).
21. T. Ono and Y. Imai, *Methods Enzymol.* **110**, 375 (1985).
22. S. Yamamoto and K. Bloch, *J Biol. Chem* **245**, 1670 (1970).
23. D. Lee and M. Smith, *Exp. Parasitol.* **16**, 392 (1965).
24. D. R. Sherman and D E. Goldberg, unpublished results.
25. M. Dresselhaus and L. Acker, *Getreide Mehl Brot* **28**, 137 (1974).
26. H. Davenport, *Proc R Soc. London Ser. B* **136**, 271 (1949).

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Cryobiological Preservation of *Drosophila* Embryos

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The inability to cryobiologically preserve the fruit fly *Drosophila melanogaster* has required that fly stocks be maintained by frequent transfer of adults. This method is costly in terms of time and can lead to loss of stocks. Traditional slow freezing methods do not succeed because the embryos are highly sensitive to chilling. With the procedures described here, 68 percent of precisely staged 15-hour Oregon R (wild-type) embryos hatch after vitrification at −205°C, and 40 percent of the resulting larvae develop into normal adult flies. These embryos are among the most complex organisms successfully preserved by cryobiology.

Worldwide, some 10,000 to 20,000 lines of mutant *Drosophila* are maintained by frequent and costly transfer of breeding stocks. Such stock maintenance can result in genetic drift or the loss of stocks because of poor reproductive capacity, accident, mix-up, or contamination. Consequently, the ability to cryobiologically preserve such stocks indefinitely, as is done with stocks of other organisms such as the mouse and nematode *Caenorhabditis elegans*, would be of substantial value. A priori, one might consider embryos at the early stages better candidates for preservation than those at the later stages because of their simpler structure. We and Steponkus and co-workers found independently, however, that early embryonic stages of *Drosophila* were far poorer candidates for cryopreservation than 12- to 13-hour embryos, even though the latter contain about 50,000 cells (*1*) that are well differentiated into organ systems (hatching occurs at ~21 hours at 24°C). In contrast, mouse embryos are generally frozen at the 8-cell stage (*2*), and *C. elegans* larvae at the 558-cell stage (*3*).

The survival of cells exposed to cryogenic temperatures is critically dependent on (i) the avoidance of intracellular ice and (ii) the presence of intracellular molar concentrations of protective solutes. To fulfill these two criteria, cells must be permeable both to water and the cryoprotectant. Unfortunately, *Drosophila* are permeable to neither. The embryos are surrounded by a vitelline membrane that is rendered impermeable by waxes (*4*), and the larvae and adults possess a cuticle that is impregnated with lipids (*5*). To achieve cryobiological preservation, these barrier compounds must be removed without injury. Following the approach of Limbourg and Zalokar (*4*), Lynch *et al.* (*6*) and subsequently our group (*7*) developed methods for permeabilizing the vitelline membrane of 12- to 14-hour embryos. Our protocol exposes embryos for precise times (90 to 110 s) to heptane containing low (0.3 or 0.4%) and precise concentrations of alcohol (1-butanol). Our operational criterion of permeabilization is that the embryos stain ruby red or dark pink after 5 min in a 0.1% solution of the dye rhodamine B. The functional criteria are that they shrink in hyperosmotic (0.75 M) sucrose in D-20 *Drosophila* medium (*8*) in a few minutes (indicating permeability to water) and that they initially shrink and subsequently return to normal volume in about 20 min in 2 M solutions of ethylene glycol in D-20 (indicating permeability to that cryoprotective solute). On the basis of the staining criterion, the heptane-butanol procedure permeabilizes about 90% of 12-hour embryos. Over 80% of the embryos survive (hatch). This permeabilization procedure, however, is deleterious to 3-hour embryos.

The standard approach to successful

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cryopreservation is to cool cells slowly enough so that intracellular water flows osmotically to the exterior and freezes there rather than intracellularly (9). Myers *et al.* (*10*) have calculated that such a rate for *Drosophila* embryos is <1°C per minute. However, such a slow cooling approach is not feasible for *Drosophila* because of the second formidable barrier to their cryopreservation: The embryos are highly chill sensitive even in the absence of ice and become increasingly so as the temperature falls toward −25°C (*11*, *12*). Indeed, if unpermeabilized 12-hour embryos are cooled at <1°C per minute, nearly all of them are killed before reaching that temperature. Three- to 6-hour embryos are even more chill sensitive, at least at 0°C (*11*).

The acceleration in chilling injury with lowered temperature, if maintained below −25°C, suggested that the only way to avoid such lethal consequences is to outrace them by cooling (and warming) the embryos at 20,000°C per minute (*11*). Unfortunately, at the usual concentrations (1 to 1.5 M) of cryoprotectants, such cooling rates would guarantee lethal intracellular freezing. The only way to avoid such intracellular freezing is to introduce very high concentrations (≥50 wt%) of glass-inducing solutes in and around the embryos, concentrations that are high enough both to induce vitrification during cooling and to prevent devitrification (crystallization) during warming and yet not be toxic. Steponkus *et al.* (*13*) reported that ethylene glycol can act as the glass-inducing solute when added in two steps in a manner similar to Rall's approach for mouse embryos (*14*). In Steponkus' procedure: (i) The embryos are first fully permeated by 2.1 M ethylene glycol at 22°C. (ii) The intracellular concentration of ethylene glycol is then raised to high levels by osmotically dehydrating the embryos by a 5- to 8-min exposure to 8.5 M ethylene glycol at 0°C. After this dehydration step, the embryos were cooled at ~25,000°C per minute to −205°C in nitrogen slush (which is obtained by applying a vacuum to liquid nitrogen). Upon rapid warming, 18% of 13-hour embryos hatched; however, only 3% of the resulting larvae developed into adult flies (*13*).

We have subsequently confirmed these results (*15*). Our hatching rate for 12-hour embryos averaged 12%, and 5% of the resulting larvae developed into adults. With respect to the cause of injury, we have found survival to be far more sensitive to the warming rate than to the cooling rate. Thus, decreasing the warming rate from 100,000° to 2,000°C per minute dropped the survival from 12 to 0%, whereas a similar lowering of the cooling rate had much less effect. This asymmetry strongly suggests that chilling injury is less a contributor to loss in viability than is damage from the devitrification of critical portions of vitrified cytoplasm during warming, probably because those compartments contain insufficient ethylene glycol. Measurements by differential-scanning calorimetry lend support to this view (*16*).

We (*7*, *11*) originally selected 12-hour embryos (stage 14) [figure 8 in (*17*)] for study because a new permeability barrier that is refractory to butanol-heptane (probably lipids in the cuticle) appears between 14 and 16 hours of development. The consequence of this barrier is that few (or no) older embryos survive exposure to −200°C (*7*, *11*). However, recently both we (*15*) and Steponkus and Caldwell (*18*) have found that as embryos age from 12 to 15 hours, hatching survivals after cryogenic treatment increase substantially, in our case from 12 to about 30% and in their case from 18% to 49 to 55%. They also reported that with minor changes in permeabilization procedure, 11% of the larvae from cryogenically treated embryos developed into adults.

Previously we controlled the developmental stage of the embryos at the time of the experiment by controlling the time and temperature at which they were held after a 1-hour collection period at 24°C in late morning (*7*, *15*). To obtain stage 14 embryos early the following morning, the eggs were held 20 hours at 17.5°C. We computed the developmental age of the embryos at that time to be 12 hours, based on the data in (*19*) showing the developmental rates at 17.5°C to be half of that at 24°C. To obtain older embryos, we continued the incubation at either 17.5° or 24°C and calculated the age on the basis of the same relative developmental rates. Steponkus *et al.* equated developmental stage with the number of hours elapsing at 25°C between collection and the onset of an experiment (*13*). A problem with this method is that slight experiment-to-experiment differences in temperature may result in cryobiologically significant differences in developmental stage at the time of the onset of freezing. For example, a 1°C shift in temperature from 18°C will change the rate of development by 15%.

We report here that with a developmentally more precise way of staging the embryos and modifications in the posttreatment culture techniques, 60 to 75% of vitrified embryos hatched, and a mean of 40% of the resulting larvae developed into fertile adults. The overall efficiency (number of adults per number of embryos vitrified) was thus increased to 25%: a 50-fold increase over the previous ≤0.5% figure (*13*, *15*). Such a survival percentage is equivalent to that obtained with mouse embryos cryobiologically preserved under optimal conditions (*2*). The greater precision for staging the embryos was achieved by altering the procedure from one based on the time and temperature of incubation after embryo collection (*15*) to one based on time at a fixed temperature (24.5° ± 0.5°C) from a clearly identifiable later developmental stage; specifically, the point at which 50% of the embryos are at stage 14 and 50% are at stage 15 [as defined by figure 8 in (*17*)]. At ×25 magnification, stage 14 embryos contain a central spherical dark mass (the gut), which is transformed in stage 15 to resemble three coils of a helical spring. In our new procedure, samples of freshly layered embryos were incubated for 20 hours at 17.5°C as before (at which time 90% or more are generally at stage 14); then the outer obscuring chorion was removed from one sample by standard treatment with 2.5% hypochlorite (usually followed by permeabilization in butanol-heptane). All samples were held at 24.5° ± 0.5°C until there was close to a 1:1 ratio of stage 14 and stage 15 embryos in the dechorionated sample (a point that can be determined within ±5 min). That point was defined as zero time. Successive samples were then dechorionated and permeabilized at precise times from zero. After permeabilization, the samples were incubated on D-20 for 45 min at 24°C (in order to permit recovery from permeabilization stress), exposed to 2 M and 8.5 M ethylene glycol, and then cooled at ~100,000°C per minute to approximately −205°C. The embryos were warmed at similar rates a few seconds later and checked for survival. (In other frozen cells or organisms, including mouse embryos, the time the organism is maintained at −200°C is irrelevant.)

Figure 1 shows the survival of the treated embryos as a function of time from zero at the completion of permeabilization and the 45-min recovery period, when zero is defined as indicated above. The time −1.0 hours corresponds to the 12-hour embryos used in our previous studies (*7*, *11*, *15*). Hatching percentages rose dramatically from 12% at −1.0 hours to 68% at +2.4 hours and then fell equally dramatically to 10% by +3.9 hours. In a separate experiment, we determined that when embryos were incubated at 24.5° ± 0.5°C after collection, the time defined as zero occurred 12.5 hours from the midpoint of the 1-hour collection. That is the basis for the second abscissa in Fig. 1. Development times cited henceforth refer to these two abscissas.

The two sets of time in Fig. 1 are the times after the completion of permeabilization and the 45-min incubation but before the subsequent exposure to ethylene glycol

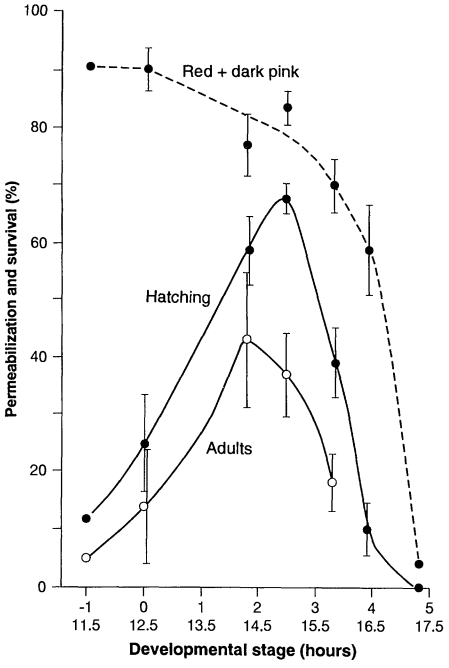

Fig. 1. Relation between the developmental stage of *Drosophila melanogaster* Oregon R-P2 embryos and (i) the percentage that are permeabilized by heptane-butanol (dashed curve) and (ii) the percentage that survive subsequent very rapid cooling to −205°C and very rapid warming (solid curves). Zero hours on the upper abcissa is the time at which about half the embryos are stage 14 and half are stage 15. At 24°C that time occurs about 12.5 hours after egg laying (lower abscissa). The times plotted include the ~30 min to effect permeabilization and the 45-min recovery period. Procedural details are given in (*21*). The middle (solid) curve gives the percent hatching of samples after ultrarapid cooling to −205°C and warming in five replicate experimental samples (three experiments for zero time). The leftmost and rightmost solid points are from (*15*). The percent hatching of controls subjected to dechorionation only and to dechorionation plus permeabilization prior to +1.6 (*14*) hours were 96 ± 1 and 95 ± 1%, respectively. The percent hatching of +1.8- and 2.4-hour (14.3- and 14.9-hour) embryos after exposure to ethylene glycol but not vitrified was 85 ± 3%. The bottom (solid) curve shows the percentage of larvae from vitrified embryos that developed into adults. The percentage of larvae from permeabilized control embryos that developed into adults was 90 ± 4% (n = 3). n = number of trials. Error bars are standard errors with n = 5 (n = 3 for zero time).

and rapid cooling to −205°C. Table 1 shows the distribution of morphological stages at the various times from zero both before and after the incubation. Development continues during that 45-min recovery period. Peak hatching percentages after cryogenic exposure occurred when the ratio of early and mid-stage 16 embryos was about 1:1 at the completion of permeabilization. We define early stage 16 as that depicted in figure 7 of (*17*). Mid-stage 16 lies between that point and the appearance of air-filled trachea.

The dashed curve in Fig. 1 shows the percentages of permeabilized embryos (percent staining ruby red or dark pink with rhodamine B) as a function of time from time zero. (Staining was carried out after the 45-min incubation.) The fraction staining red or dark pink dropped from 90% at zero time to about 80% at +2.4 hours, the time yielding the peak hatching survival of 68%. By +3.9 hours, the percentage permeabilized dropped to 58% and became more variable; the percentage hatching dropped to 10%. After another hour of development, both the percentage of embryos that become permeabilized and the percentage that survive cryogenic exposure drop to or near zero (*15*).

Because permeabilization was initiated 1.2 hours prior to rhodamine staining (~30 min to permeabilize plus a 45-min incubation), we interpret these data to mean that portions of the cuticle begin to become refractory to permeabilization by butanol-heptane between +1.5 and +2.5 (14 and 15) hours of development. Consequently, compartments in the embryo do not attain sufficiently high concentrations of ethylene glycol to maintain the vitreous state during cooling and warming. Because the inability to survive cryogenic exposure occurs at an earlier developmental stage than the complete loss of staining, it appears that only part of the cuticle has failed to permeabilize to result in poor survival.

The bottom curve in Fig. 1 shows the percentage of the resulting larvae that developed into adult flies as a function of the developmental stage prior to the initiation of glycol treatment and cooling to −205°C. The curve parallels the hatching curve. As development progressed from −1 (11.5) to +1.8 (14.3) hours, the percentage of adults increased from 5 to 43% and then decreased to 37% in +2.4- (14.9-) hour embryos and to 18% in +3.3- (15.8-) hour embryos. The developmental time producing the peak percentage of adults appears to be 0.7 hours earlier than that producing peak hatching. In both cases, the overall efficiency (percentage of cryopreserved embryos developing to adult flies, that is, the product of the hatching and adult curves) is 25%. In five replicates to date, males constituted 49.8 ± 5% of the 162 adults derived from embryos vitrified at +1.8 and +2.4 hours (the developmental stages yielding the highest survivals). To date, 58 out of 62 individually tested females were fertile, and each fertile female produced an average of 50.3 second-generation adults in 12 days, of which 49.4% were female.

We ascribe most of the improvement in adult survival over that in (*13*) and (*15*) to the three- to sixfold increase in hatching survival at the optimal stage. That is, the factors that cause hatching percentages to be low inflict damage on most larvae, and such damage prevents subsequent development to adulthood. Some of the improvement in survival rates observed here may be due to slight modifications in culture procedures from (*15*). To determine the hatch rate, each Nuclepore filter used to support embryos during processing was placed on 1 ml of 3% agar in D-20 in a 35-mm Falcon (3001) dish to which had been added 0.06 mg of yeast [previously filters were floated on liquid D-20 without yeast (*15*)]. Then, as in (*15*), the dishes were incubated ~24 hours in a sealed box at 24° to 25°C in near 100% relative humidity.

To determine the percentage of larvae that developed into adult flies, the portions of agar that held larvae often along with the support filter were transferred to a 15 by 95 mm shell vial containing a 1:3 (w/w) mixture of Carolina Biological Supply 4-24 Drosophila medium and water. Additional yeast suspension was added then and 2 days later as in (*15*), and the vials were incubated at 24° to 25°C at 60 to 65% relative

Table 1. Distribution of *Drosophila* embryo developmental stages.

Run	Embryo age from time zero* (hours)	Before or after 45-min incubation†	Percentage in stage			
			14	15	Early 16	Mid 16
0	0 = 12.5‡	After	54 ± 4	45 ± 4	1 ± 1	0
1	0 + 1.0	Before	2 ± 2	39 ± 27	50 ± 26	9 ± 5
	0 + 1.8‡	After	0	3 ± 2	62 ± 13	35 ± 14
2	0 + 1.6	Before	0.5 ± 0.5	2 ± 1	50 ± 15	47 ± 16
	0 + 2.4‡	After	0	0	21 ± 13	79 ± 13
3	0 + 2.6	Before	0	0	8 ± 2	92 ± 2
	0 + 3.3‡	After	0	0	2 ± 1	98 ± 1
4	0 + 3.2	Before	0	0	2 ± 1	98 ± 1§
	0 + 3.9‡	After	0	0	0.3 ± 0.3	99.8§

*See text. †A 45-min recovery period at 24°C after permeabilization. ‡Developmental stage times plotted in Fig. 1. §Includes a small percentage of late stage 16 embryos defined as these exhibiting air-filled tracheal tubes.

humidity for 12 to 14 days [1 to 2 days longer than in (*15*)] or until no additional flies emerged.

The current optimal procedures produce overall survivals that constitute effective and practical cryobiological preservation of the Oregon R strain. Although it remains to be determined whether they will also be effective in preserving embryos from mutant lines, the major developmental processes are completed by stage 16, and consequently we expect that most laboratory strains will tolerate these vitrification procedures as well as Oregon R does. An advantage of defining the optimum developmental time for cryopreservation in relation to the time at which the ratio of stages 14 to 15 is 1:1 is that it will automatically compensate for any strain-to-strain differences in development rate. The approach may also be applicable to the cryopreservation of embryos from other diptera like the housefly or mosquito, which have developmental rates very different from that of *Drosophila* (*20*).

We believe our findings have more general implications for cryobiology. The optimal developmental stages being frozen are probably the most complex systems that have been cryobiologically preserved. The embryos are highly differentiated into tissues and organs including muscle and nerve, which indicates that differentiated multicellularity is not a barrier to cryopreservation per se. The findings also represent perhaps the first case in which vitrification procedures are required to obtain survival. From the mechanistic point of view there remains the question of why *Drosophila* survival is so critically dependent on developmental stage. Older embryos may vitrify more readily than younger embryos, or possibly they tolerate the presence of ethylene glycol or small amounts of ice better than younger embryos, perhaps because the critical steps of dorsal closure and head involution are completed. The answers could be important in determining the extent to which the general strategies described prove applicable to other non-mammalian eggs and embryos, most of which have not been successfully cryobiologically preserved.

REFERENCES AND NOTES

1. K. V. Anderson and J. A. Lengyel, *Dev. Biol.* **82**, 127 (1981).
2. D. G. Whittingham, S. P. Leibo, P. Mazur, *Science* **178**, 411 (1972); U. Schneider and R. R Maurer, *Biol. Reprod.* **29**, 121 (1983).
3. J. E. Sulston, E. Schierenberg, J. G. White, J. N. Thomson, *Dev. Biol.* **100**, 64 (1983); M. Edgley, *Caenorhabditis* Genetics Center Technical Note (1988).
4. B. Limbourg and M. Zalokar, *Dev. Biol.* **35**, 382 (1973).
5. N. F. Hadley, *Sci. Am.* **254**, 104 (July 1986).
6. D. V. Lynch *et al.*, *Cryobiology* **26**, 445 (1989).
7. P. Mazur, K. W. Cole, A. P. Mahowald, *ibid.* **29**, 210 (1992).
8. G. Echalier and A. Ohanessian, *In Vitro* **6**, 162 (1970); M. Zalokar and P. Santamaria, in "Syllabus of experimental methods in *Drosophila* embryology," EMBO Workshop, Gif-sur-Yvette, France, 1977.
9. P. Mazur, *Cell Biophys.* **17**, 53 (1990).
10. S. P. Myers, R. E. Pitt, D. V. Lynch, P. L. Steponkus, *Cryobiology* **26**, 472 (1989).
11. P. Mazur, U. Schneider, A. P. Mahowald, *ibid.* **29**, 39 (1992).
12. S. P. Myers, D. V. Lynch, D. C. Knipple, S. P. Leibo, P. L. Steponkus, *ibid.* **25**, 544 (abstr.) (1988); S. P. Myers and P. L. Steponkus, *ibid.* **27**, 651 (abstr.) (1990).
13. P. L. Steponkus *et al.*, *Nature* **345**, 170 (1990).
14. W. F. Rall, *Cryobiology* **24**, 387 (1987).
15. P. Mazur, K. W. Cole, P. D. Schreuders, A. P. Mahowald, *ibid.*, in press.
16. P. D. Schreuders, P. Mazur, B. Sales, *ibid.* (abstr.), in press; V. L. Bronshteyn and P. L. Steponkus, *ibid.* (abstr.), in press.
17. E. Wieschaus and C. Nusslein-Volhard, in *Drosophila—A Practical Approach*, D. B. Roberts, Ed. (IRL Press, Oxford, 1986), pp. 199–227.
18. P. L. Steponkus and S. Caldwell, *Cryobiology* (abstr.), in press.
19 M. Ashburner and J N. Thompson, Jr., in *The Genetics and Biology of Drosophila*, M. Ashburner and T. R. F. Wright, Eds. (Academic Press, London, 1978), vol. 2A, pp. 1–109.
20. J. M. Strong-Gunderson and R. A. Leopold, *Environ. Entomol.* **18**, 756 (1989); L. N. Raminani and E. W. Cupp, *J. Insect Morphol. Embryol.* **4**, 517 (1975).
21. Permeabilization was effected by successive exposure of embryos on 25-mm Nuclepore or Poretics polycarbonate filters to 2.5% hypochlorite to remove the chorion, a thorough water wash, isopropanol treatment, air-drying for 2 min to remove the isopropanol, and a mixture of 0.3% 1-butanol in *n*-heptane for 90 s, followed by a brief chase with pure heptane. Details are given in (*7*) and (*15*). The duration of the air-drying and the butanol/heptane steps are critical. The filters were rinsed several times in D-20 and then incubated 45 min at 24° to 25°C. The percentage of permeabilized embryos was defined as the percentage that stained red or dark pink after a 5-min exposure at 23°C to 0.1% rhodamine B in D-20. [The leftmost and rightmost points of the dashed curve are from (*15*).] The embryos on the filters were then exposed to 2 M ethylene glycol at room temperature (23°C) for 30 min and then to 8.5 M ethylene glycol plus 10% (w/v) polyvinyl pyrrolidone (Plasdone C-30) for 4.5 to 5.5 min at 5°C. The next step was to abruptly plunge the filters into a mixture of solid and liquid nitrogen (nitrogen slush) at approximately −205°C and then 10 to 30 s later to abruptly plunge the filters into 0.75 M sucrose in D-20 at 23°C, hold them in that solution for 2 min, and then transfer them into D-20. Details of and reasons for these various steps are given in (*15*).

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Cellular Proteins Bound to Immunodeficiency Viruses: Implications for Pathogenesis and Vaccines

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Cellular proteins associated with immunodeficiency viruses were identified by determination of the amino acid sequence of the proteins and peptides present in sucrose density gradient–purified human immunodeficiency virus (HIV)–1, HIV-2, and simian immunodeficiency virus (SIV). β2 microglobulin (β_2m) and the α and β chains of human lymphocyte antigen (HLA) DR were present in virus preparations at one-fifth the concentration of Gag on a molar basis. Antisera to HLA DR, β_2m, as well as HLA class I precipitated intact viral particles, suggesting that these cellular proteins were physically associated with the surface of the virus. Antisera to class I, β_2m, and HLA DR also inhibited infection of cultured cells by both HIV-1 and SIV. The specific, selective association of these cellular proteins in a physiologically relevant manner has major implications for our understanding of the infection process and the pathogenesis of immunodeficiency viruses and should be considered in the design of vaccines.

Studies with subunit vaccines have shown that immunizations with viral envelope antigens alone are sufficient to elicit protective immunity against SIV or HIV (*1*). The recent observation (*2*) that macaques immunized with uninfected human cells were protected against challenge with SIV grown in human cells raised the possibility that immune responses to cellular antigens might also be involved in protection. However, the putative cellular antigens that may be stimulating the protective response have not been identified, and the mechanism of protection is unclear.

To identify the specific cellular antigens associated with immunodeficiency viruses, we purified and sequenced proteins from preparations of HIV-1, HIV-2, and SIV.