Gilbert, J. B. Posner, Eds. (Hall, Boston, 1980), p. 30; *Invasion Metas.* **3**, 193 (1983).
14. G. A. Turner, *Invasion Metas.* **2**, 197 (1982).
15. A. F. Chambers, R. P. Hill, V. Ling, *Cancer Res.* **41**, 1368 (1981); L. Weiss, in *Tumor Invasion and Metastasis*, L. A. Liotta and I. R. Hart, Eds. (Nijhoff, Boston, 1982), p. 81.
16. A. Raz and A. Ben-Ze'ev, *Science* **221**, 1307 (1983).
17. H. B. Bosmann and A. Lione, *Biochem. Biophys. Res. Commun.* **61**, 564 (1974).

18. We thank R. F. Kuba and M. Naik for technical assistance. B16F1 and B16F10 cells were the kind gift of I. J. Fidler. Supported by the National Cancer Institute of Canada, the Medical Research Council of Canada, and the Ontario Cancer Treatment and Research Foundation.
* Present address: Ontario Cancer Foundation, London Clinic, London, Ontario, Canada N6A 4G5.

5 December 1983; accepted 29 February 1984

The Respiratory Sinus Arrhythmia: A Measure of Cardiac Age

Abstract. *A method developed for quantifying respiratory sinus arrhythmia (RSA) during voluntary cardiorespiratory synchronization relies on computer-assisted rhythmometric cosinor analysis of instantaneous heart rate data. The RSA was present in all subjects tested, even those at advanced ages. The amplitude of the RSA falls approximately 10 percent per decade. An individual with a transplanted heart and one with severe diabetic neuropathy each had resting RSA values that were normal for their ages. The shape and amplitude of the RSA during voluntary cardiorespiratory synchronization may reflect the suppleness of the heart and its response to rhythmically changing intrathoracic pressure and the subsequent ebb-and-flow of venous return. Our technology allows objective quantitative assessment of the biologic age of the heart and also the effect of any drug, disease, or behavior that affects the RSA.*

In 1733, Hales observed that changes in blood pressure and pulse were related in a regular manner to the respiratory pattern in the horse (*1*). In 1846, Ludwig's invention of the kymograph allowed his observation of the regular quickening of pulse with inspiration and slowing with expiration in the dog (*2*). Medical students have been taught for more than a century that this regular irregularity of heart beat disappears with advancing age (*3*). Since few biologic phenomena disappear abruptly, it seemed to us that the inexactitude of clinical methods for detecting the respiratory sinus arrhythmia (RSA) might be responsible for its apparent disappearance. Furthermore, any cardiovascular physiologic end point that changes predictably with advancing age is of potential major interest.

Quantitative statistical analysis of this phenomenon has been made possible by two discoveries. In 1963, a voluntary coupling system that allows the subject to couple breathing pattern to heart rate (*4–6*) was developed. The coordination of pulse and breathing allows straightforward analysis of resultant instantaneous heart rate data. Since prior statistical evaluations of the RSA have relied upon the assessment of heart rate variance in the unsynchronized state, only very gross, usually pharmacologically induced, differences have been perceptible (*7, 8*). In 1972, development of the single cosinor method of analysis made accurate quantification and statistical analysis of rhythmic biological functions practical (*9*). Spectral analysis of heart rate periodicity and the variance of periods within a window encompassing the respiratory frequency has been the most widely used alternative methodology (*10, 11*).

Modern microcomputer technology has allowed us to investigate the reproducibility of this physiologic parameter and to quantify the effect of advancing age. We studied a group of 25 healthy subjects, ranging in age from 20 to 82, who visited the cancer detection clinic for routine screening examination. We also investigated RSA mechanisms in patients selected because of their unique cardiovascular anatomy or disease state.

The principal features of this system include a pulse sensor; a current discriminator and clock to count and time successive electrical impulses generated by the pulse transducer that correspond to sensed heartbeats and determine an instantaneous heart rate; and driver circuits to relay the desired ratio pattern of visual and auditory signals displayed through a cathode ray tube that advise the subject to inhale and exhale for each of the preselected number of heartbeats. This system is closed when the subject voluntarily breathes according to the transmitted instructions. The subject's ability to follow visual signals is quantitatively verified by a mouthpiece-mounted thermistor which detects breathing (Fig. 1).

All data in this report were obtained using the ratio of two beats for inspiration followed by three beats for expiration, but any desired ratio may be studied in this way. The beat-to-beat interval

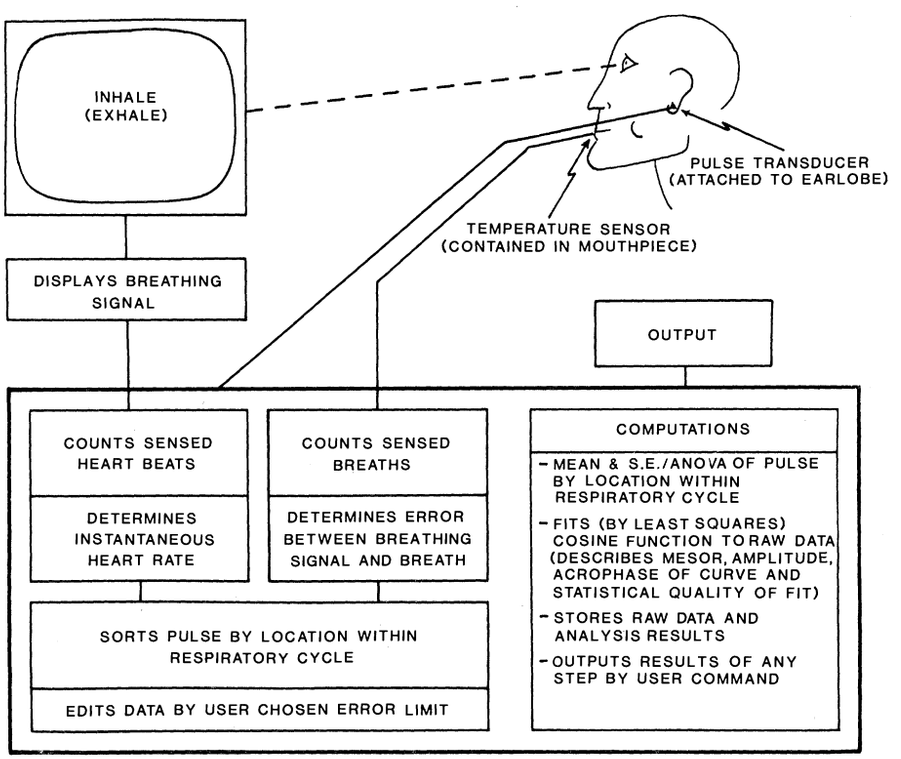

Fig. 1. The necessary components and basic function of the Sine-o-graph pulse monitor, including a pulse sensor to count beats and determine instantaneous heart rate, a respiration sensor for discrimination of inhalation and exhalation and sorting with pulse, interactive software and display of inhale and exhale signals, a statistical package, and a unit for hard copy output.

translated to instantaneous heart rate is the experimental datum. These data were sorted and stored according to their exact position in the five-beat cycle and then analyzed. The first pulse corresponds to the interval between the first and second beats of the inspiration phase of the respiratory cycle.

Biologic functions are rhythmic within many time frames. The RSA represents an ultradian or high-frequency biologic rhythm. All data are analyzed and reported independent of time in terms of the arbitrarily preselected and standardized period length of five heart beats. The data are expressed as average instantaneous heart rate during each of the five beats of this five-beat rhythm. If the average pulse during 1 minute is 75 beats per minute, 15 continuous pulse cycles of a five-beat rhythm are evaluated in that minute. Single cosinor analysis was used to investigate each series of pulse data for five-beat rhythmicity. This analysis involves a least-squares regression fit of a cosine curve to the data points. The fitted curve describes the shape of this rhythm by providing the mean pulse value, the amplitude, and the timing of the peak with their respective 95 percent confidence intervals. The population mean cosinor analysis was used to investigate whether the rhythm characteristics of individual data series were consistent (*9*). Classical univariate and multivariate mean analysis and Bingham testing were used to complement cosinor analysis (*12*). "Statistical significance of a rhythm" is claimed only when the cosine model fits well ($P < 0.05$) and the analysis of variance of pulse by position within the respiratory cycle demonstrates a difference ($P < 0.05$). Linear regression analysis was used either separately or in combination with cosinor analysis to inspect the data for trends.

Twenty-five presumably healthy subjects of both sexes, aged 20 to 82, were studied at 7 a.m. and again at 9 a.m., about 30 minutes after venipuncture. The heart rate, amplitude, and phasing of the RSA showed relative stability. The degree of intraindividual variability in these parameters was much smaller than the amount of interindividual variability (Table 1).

Each individual in every age-defined population had a highly statistically significant five-beat rhythm (analysis of variance, $P < 0.01$; cosine fit $P < 0.001$) (data not shown). An age-dependent fall in amplitude (slope, 10 percent per decade; $r = 0.49$, $P < 0.01$) and shift in timing of peak heart rate from later to earlier beats in the five-beat cycle (slope, 0.1 beat per decade; $r = 0.47$, $P < 0.01$) were found with advancing age (Table 2). Extension of the regression line describing the age-dependent fall of amplitude shows that the maximum degree of rhythmic variation (peak-trough difference) of pulse rate would be expected to be about 24 percent of the heart rate, whereas the amplitude would be expected to disappear at ~ 120 years of age.

The 25 subjects were arbitrarily grouped into one of four age categories, each spanning approximately 15 years:

Table 1. Intraindividual comparison of five-beat sinus arrhythmia. Values in parentheses are 95 percent confidence intervals. While two-way analysis of variance by time and beat corroborated the significant effect of beat (P of five-beat rhythm), there was no significant effect of time of testing (F test; P value for 7 a.m. versus 9 a.m.) on the five-beat sinus arrythmia. Rhythm characteristics tested in all combinations showed no significant differences for 7 a.m. or 9 a.m.

Time of testing	Number of subjects	Mean (beats per minute)	Amplitude (beats per minute)	Timing of peak rate, beats 1 to 5	P of five-beat rhythm
		Absolute data			
7 a.m.	25	73.97 (69.90, 78.02)	2.12 (1.56, 2.68)	2.00 (1.80, 2.17)	< 0.001
9 a.m.	25	72.49 (68.15, 76.81)	2.34 (1.74, 2.94)	1.86 (1.74, 1.95)	< 0.001
F test, d.f.(1,48)		0.26	0.32	1.87	
P value		0.61	0.57	0.18	
		Data expressed as percent of mean			
7 a.m.	25	100.0 (98.5, 102.3)	3.0 (2.2, 3.8)	1.97 (1.78, 2.14)	< 0.001
9 a.m.	25	98.7 (96.6, 100.8)	3.2 (2.4, 4.0)	1.84 (1.72, 1.94)	< 0.001
F test, d.f.(1,48)		1.54	0.20	1.66	
P value		0.22	0.65	0.20	

Table 2. Age comparison of five-beat sinus arrhythmia. Values in parentheses are 95 percent confidence intervals. Two-way analysis of variance by age and beat number corroborated the significant effect of age on the five-beat sinus arrythmia.

Age (years)	Number of profiles	Mean (beats per minute)	Amplitude (beats per minute)	Timing of peak rate, beats 1 to 5	P of five-beat rhythm
		Absolute data			
20 to 35	6	70.55 (61.95, 79.16)	3.82 (2.46, 5.18)	2.06 (1.78, 2.26)	< 0.001
35 to 50	10	85.46 (80.57, 90.35)	2.93 (2.24, 3.62)	2.07 (1.89, 2.24)	< 0.001
50 to 65	20	68.85 (65.79, 71.91)	2.08 (1.65, 2.51)	1.86 (1.72, 2.01)	< 0.001
65 to 82	14	71.89 (65.69, 78.09)	1.38 (0.44, 2.32)	1.68 (1.18, 2.07)	< 0.001
F test, d.f.(1,48)		9.72	7.10	2.71	
P value		< 0.001	< 0.001	0.06	
		Data expressed as percent of mean			
20 to 35	6	98.7 (89.7, 107.7)	5.4 (3.0, 7.8)	2.06 (1.78, 2.26)	< 0.001
35 to 50	10	99.7 (96.3, 103.5)	3.4 (2.6, 4.2)	2.07 (1.89, 2.24)	< 0.001
50 to 65	20	99.9 (97.8, 102.0)	3.0 (2.4, 3.6)	1.86 (1.72, 2.01)	< 0.001
65 to 82	14	99.9 (97.5, 102.3)	2.2 (0.9, 3.5)	1.68 (1.22, 2.00)	< 0.001
F test, d.f.(1,48)		0.09	5.20	3.00	
P value		0.96	< 0.01	0.05	

20 to 35, 36 to 50, 51 to 65, and 66 to 82. The amplitude (half the peak-trough difference) of the RSA decreases from four beats per cycle in the youngest group to just over 1.3 beats per cycle in the oldest age group. Figure 2 depicts reconstructed curves representing the age-dependent changes in these four populations. Classical multivariate analysis and the Bingham testing (*12*), an analysis of variance technique comparing rhythm parameters singly and jointly, confirm that the pulse data of the four age-defined populations differ [amplitude: d.f.(3,46), $F = 5.2$, $P < 0.01$; phase: d.f.(3,46), $F = 3.0$, $P < 0.05$]. When extensively tested by multivariate techniques, the amount of heart rate and RSA amplitude variability due to age class or interindividual difference was always far greater than that secondary to intraindividual differences.

A 44-year-old male patient with a transplanted heart extirpated from a 35-year-old male motorcycle accident victim was studied shortly after removal of all chest tubes within a week after surgery. He felt well, and results of the cardiorespiratory examination were normal. RSA was present in this patient (analysis of variance, $F = 31.7$, $P < 0.001$; cosinor fit, $P < 0.001$). The amplitude of the rhythm was 1.7 ± 0.2 beats per minute. His peak value occurred at the usual position of the five-beat cycle. Figure 3 shows the relation of the mean and standard errors of pulse and the fitted curve of this man's RSA to the data and curve from several age-matched control subjects. A two-way analysis of variance of the mean pulse change examined by beat number in the control subjects versus that in the transplant patient revealed a highly statistically significant beat-dependent (RSA) pulse difference ($F = 19$, $P < 0.001$) but no difference between the pulse pattern (RSA) of this patient and that of the control subjects ($F = 0.2$, $P < 0.66$).

A 47-year-old white female with a long history of diabetes mellitus that had been poorly controlled for at least 20 years was also studied. The patient had mild orthostatic hypotension; intermittent nausea, vomiting and diarrhea; and severe numbness of the fingers and numbness of the feet to the level of the knee. She complained of not being able "to tell where her feet are." Physical examination revealed a resting tachycardia of 116 beats per minute and a 20-mmHg drop in diastolic blood pressure upon standing, without a concomitant pulse rise. This patient had diabetic retinal and renal abnormalities, as well as severe peripheral neuropathy. An abnormal sweating and Valsalva response also showed neuropathy. Nerve conduction and an electromyogram were severely abnormal. The amplitude of evoked muscle response was markedly decreased, and distal sensory latencies were unobtainable in the right median and sural nerves. Needle electrode examination showed changes of denervation in all distal muscles tested. A sural nerve biopsy showed both light and electron microscopic changes consistent with severe degenerative changes.

Analysis of the RSA in this patient showed a five-beat rhythm (analysis of variance, $F = 22.3$, $P < 0.001$; cosine fit, $P < 0.0001$). The predictable swing of nearly four beats per minute was compared to a maximum swing of 2.6 beats per minute in an age-matched control subject. A two-way analysis of variance of pulse examined by beat and comparing the data of the diabetic subject to those of the control subjects revealed no statistically significant difference in their pulse patterns.

A large number of reports have described and attempted to explain the respiratory sinus arrhythmia. Most of these reports included pulse data without synchronization of the respiratory pattern (*13–16*). Most methods relying on power spectrum analysis of heart rate within the frequency window relevant to respiratory cycles are opaque by comparison to cosinor analysis of pulse data

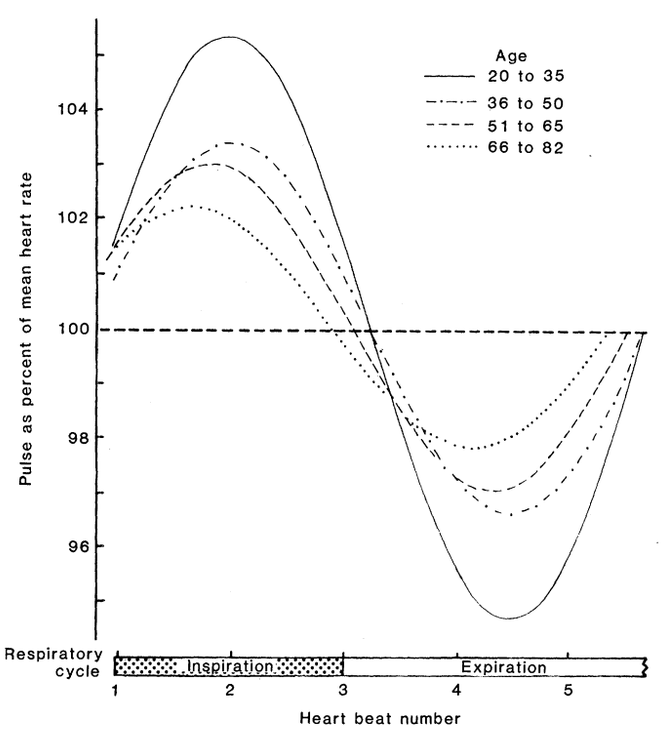

Fig. 2 (left). As aging progresses, the amplitude of this five-beat pulse RSA rhythm falls about 10 percent per decade, and the timing of the peak pulse gradually shifts from the second toward the first beat of the five-beat cycle. Raw data from which the curves are derived are depicted in Table 2.

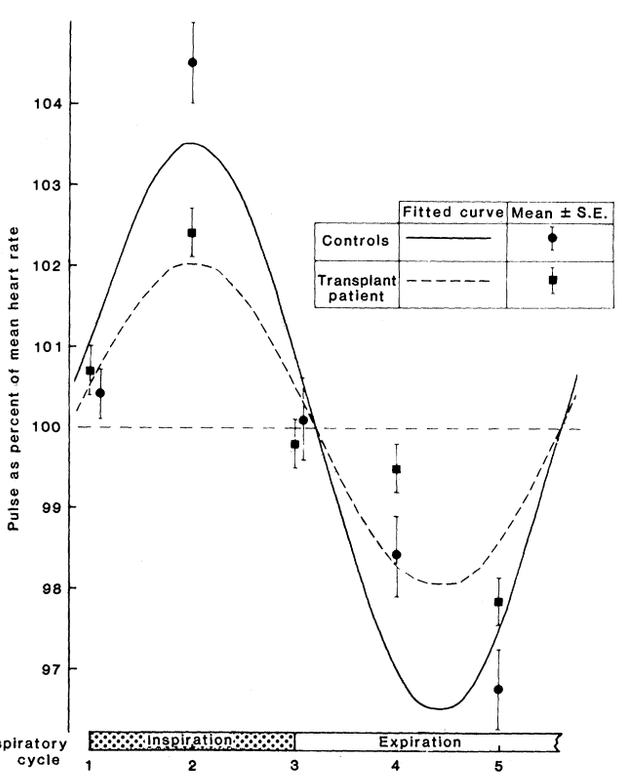

Fig. 3 (right). A statistically significant resting RSA remains in the data from a 44-year-old male, one week after cardiac transplantation (– – –). The pulse data from age-matched control subjects and our patient do not differ, as determined by analysis of variance ($F = 0.4$, $P = 0.8$).

synchronized with respiration. These spectral methods have been useful primarily for determining the effects of pharmacologic doses of potent cardiotropic drugs. Synchronization of breathing with pulse results in immensely less nonpredictable variability and a far more precise evaluation of the sinus arrhythmia.

Our curve-fitting method of assessment of the resting RSA, during voluntary cardiorespiratory synchronization, had reasonably small test-to-test variability within the same individual who had been tested at roughly the same times of day before and after venipuncture. The resting RSA amplitude is an easily measured index that correlates well with age. The timing of the peak of the function best described by the individual data also changes predictably with advancing age. These predictable relationships allow the construction of nomograms that correlate chronologic age, the RSA amplitude or timing of peak value, and "biologic cardiac age." This technology provides an objective measure of physiologic cardiovascular aging.

Data obtained on a patient after cardiac transplantation and a patient with severe neuropathy lead us to agree with the hypothesis put forward by Bainbridge (*17*) in 1920, stating that a negative pressure-dependent increase of the diastolic filling of the heart increases cardiac rate primarily through intracardiac, rather than extracardiac, sympathetic and parasympathetic reflex arcs. This is not to say that these heart rate changes cannot be overridden by sympathetic or parasympathetic discharge, by circulating chronotropes like epinephrine or norepinephrine, or by other cardioactive drugs.

Our results in patients with absolutely and relatively denervated hearts lead us to believe that the resting RSA is primarily a measure of cardiac suppleness, elasticity, or tissue compliance. For this to be proved, concurrent, more direct measurements of cardiac compliance need to be correlated with this index. Chest wall compliance also needs to be considered by measuring and correcting for respiratory volume and for the rate of and amount of negative pressure induced during inspiration.

If some basic property of the heart tissue, such as myocardial compliance, is the primary variable being measured, longitudinal assessment of cardiac status would seem to be pertinent for individuals suffering from many cardiovascular diseases and possibly in routine health maintenance. This technology will allow quantitative objective assessment of aging, as well as any drug effect, disease, or behavior that affects the RSA. The instrument may also be useful for screening drugs for cardiotoxicity or general cardioactivity.

W. J. M. HRUSHESKY
D. FADER
Departments of Medicine and Laboratory Medicine and Pathology, University of Minnesota Medical School, Masonic Cancer Center, Minneapolis 55455
O. SCHMITT
Department of Biophysics, University of Minnesota Medical School
V. GILBERTSEN
Department of Surgery, University of Minnesota Medical School

References and Notes

1. S. Hales, *Haemastaticks* (Innings and Manby, London, 1733).
2. C. Ludwig, *Archiv fur Anatomie and Physiologie und Wissenschofliche Medicin* (1847), p. 242.
3. E. Hering, *Sitzungsber. Akad. Wiss. Wien. Math. Naturwiss. Kl. Abt.* **64**, 333 (1871).
4. J. J. Almasi and O. H. Schmitt, *Ann. N.Y. Acad. Sci.* **170**, 509 (1970).
5. J. Almasi, thesis, University of Minnesota (1972).
6. O. Schmitt and J. Almasi, *IEEE Trans. Biomed. Eng.* **21**, 264 (1974).
7. S. Akselrod, D. Gordon, F. A. Ubel, D. C. Shannon, A. C. Barger, R. J. Cohen, *Science* **213**, 220 (1981).
8. B. Womack, *IEEE Trans. Biomed. Eng.* **18**, 339 (1971).
9. F. Halberg, E. A. Johnson, W. Nelson, W. Runge, R. Sothern, *Physiol. Teach.* **1**, 1 (1972).
10. S. W. Porges *et al.*, *Psychol. Bull.* **88**, 580 (1980).
11. S. W. Porges, R. E. Bohrer, G. Keren, M. N. Cheung, G. J. Franks, F. Drasgow, *Psychophysiology* **18**, 42 (1981).
12. C. Bingham, G. Arbogast, G. Cornelissen, J. K. Lee, F. Halberg. *Chronobiologia* **9**, 397 (1982).
13. G. Chess, R. Tam, F. Calaresu, *Am. J. Physiol.* **228**, 775 (1975).
14. E. Fischmann, J. Cosma, H. V. Pipberger, *Am. Heart J.* **75**, 465 (1968).
15. A. Melcher, *Acta Physiol. Scand.* **435**, 6 (1976).
16. I. Starr and C. Friedland, *J. Clin. Invest.* **25**, 53 (1946).
17. F. A. Bainbridge, *J Physiol. (London)* **54**, 192 (1920).
18. No federal, state, or private foundation funding has been used in the development or testing of this invention to date. A U.S. patent is pending. Patent applications have also been filed in the United Kingdom, Canada, Europe, and Japan. Inquiries about this instrument should be addressed to: Sine-o-graph Corp., 3538 Fremont Avenue South, Minneapolis, Minn. 55408.

21 November 1983; accepted 23 March 1984

Transient Expression of Homologous Genes in *Drosophila* Cells

Abstract. *A cloned* Drosophila *heat shock protein 22 gene was transfected into two independently established* Drosophila *cell lines. Each line carried a different heat shock protein 22 allele, distinguishable by electrophoresis of the protein. The transfected gene was not expressed at 25°C but could be induced at 36°C. In one line, two heat shock protein 22 electromorphs were synthesized.*

Cloned genes have been introduced into cultured somatic cells, unfertilized eggs, or zygotes under conditions that lead to their expression and regulation (*1*). Such transformation and transient expression systems provide a functional assay for defining DNA sequences flanking structural genes that are necessary for control of their expression (*2*). One limitation to these systems is that the introduced gene and recipient cell are seldom homologous—that is, they are usually derived from different species—because in most cases one could not distinguish qualitatively the expression of the transfected gene from the expression of the equivalent endogenous gene. As a consequence, several strategies have been developed to study the regulated expression of transfected genes. One is to use a completely heterologous system consisting of a cloned gene and a recipient cell from different species (*3*). The foreign gene, when expressed, produces an RNA and protein molecule whose presence can then be assayed directly and with no interfering signal from endogenous gene expression. A second strategy is to link physically the putative regulatory DNA sequence of a homologous gene to a heterologous structural gene (*4*). In this chimera the homologous regulatory region then drives the transcription and subsequent translation of a foreign gene whose expression can then be assayed directly. This approach introduces some homology. A third approach is to use a recipient cell that contains a mutationally defective or functionally inactive gene so that the expression and regulation of a gene introduced by transfection can be measured unambiguously (*5*). Such systems are limited by the availability of the necessary mutant line.

We now describe our study of homologous gene expression in a transient expression system. Although our experiments were performed with cultured *Drosophila* cells as recipients for transfection, the same principles would work for gene transfer into eggs (transformation) and would apply for any species. Our approach exploits the presence of ubiquitous genetic polymorphism, which appears in the form of electrophoretic mobility variants (*6*). We first identified two independently isolated cell lines that expressed different electrophoretic variants at some protein coding locus. The