Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1352–1356, March 1983
Cell Biology

Cold-sensitive expression of transformation by a host range mutant of type 5 adenovirus

(temperature shifts/integration pattern/RNA transcription and processing/anchorage independence/saturation density)

LEE E. BABISS, HAROLD S. GINSBERG, AND PAUL B. FISHER

Department of Microbiology, Cancer Center/Institute of Cancer Research, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Contributed by Harold S. Ginsberg, November 18, 1982

ABSTRACT **hr1, an E1a (0–4.5 map units) host range mutant of type 5 adenovirus (Ad5), transformed a cloned rat embryo fibroblast (CREF) cell line at about a 5-fold higher frequency than wild-type (wt) Ad5 when cells were cultured at 37°C. However, if the cells were infected with hr1 and maintained at 32°C morphological transformation did not occur. When infected cells were shifted from 32°C to 37°C 2 wk postinfection, the frequency of transformation by 6 wk was identical to that of cells grown continuously at 37°C, whereas cultures shifted from 37°C to 32°C 2 wk postinfection displayed a >96% reduction in morphological transformation. hr1-transformed cells had a fibroblastic morphology as contrasted with the typical epithelioid morphology of wt Ad5-transformed cells, but hr1- and wt Ad5-transformed cells had similar saturation densities, growth rates, and agar cloning efficiencies when assayed at 37°C. However, when cells transformed by hr1 at 37°C were grown at 32°C, they had a saturation density close to that of normal CREF cells and grew at a lower efficiency in agar than wt-transformed cells. DNA transfer/hybridization analysis of two hr1-transformed cloned cell lines, A2 and B3, indicated that A2 cells contained a complete integrated copy of the Ad5 genome, whereas in B3 cells only part of the Ad5 genome was integrated. RNA transfer and RNA/DNA filter hybridization analyses indicated that the types of viral messenger RNAs and the relative amounts of RNA transcribed were similar in the A2 and B3 cell lines when they were grown at 32°C and 37°C. Indirect immunofluorescence, with antisera from hamsters bearing Ad-induced tumors, indicated a temperature dependence in staining—i.e., cells grown at 37°C or shifted from 32°C to 37°C contained intense, particulate staining in the nuclear region, whereas the staining was decreased significantly in cells cultured at 32°C and in cells shifted from 37°C to 32°C. These findings indicate that the gene product affected by the hr1 mutation is cold sensitive and is essential for the expression of the characteristics that identify the transformed cell.**

Recent studies that employ host range (1, 2) and host range deletion (3) mutants of Ad5 suggest that the E1a [0–4.5 map units (m.u.)] and E1b (4.5–11.5 m.u.) transcriptional units are involved in the initiation and maintenance of stable transformation of rodent cells. Direct evidence for an involvement of this region of Ad5 in transformation also has come from experiments in which the E1a or E1a + E1b region of Ad5 DNA has been transferred into recipient cells by Ca^{2+}-mediated DNA transfection (4–6). A problem in determining the significance of the transformation data has been the low frequency of transformation and the lack of consistent results when different cell types were used (1, 3, 7). For example, Jones and Shenk (3) were unable to obtain transformation of primary rat embryo or rat embryo brain cells with the deletion mutant dl313 (a mutant with a deletion of 2,350 base pairs between 3.5 to 10.5 m.u.), whereas Shiroki *et al.* (7) obtained a similar transformation frequency with dl313 and wild-type (wt) Ad5 when they used the established 3Y1 rat cell line.

We have recently isolated a cell line (CREF) from a single cell clone of the F2408 Fischer rat embryo fibroblast cell line. These CREF cells have the great advantage in that they are transformed by wt Ad5 at >150-fold higher frequency than early passage rat embryo cells (8, 9). This report presents the results of a study of the interaction between CREF cells and hr1 virus (10), a host range mutant of Ad5 with a single base-pair deletion in E1a at position 1,055 (11, 12). Graham *et al.* (1) originally reported that hr1 was unable to transform rat embryo cells or rat embryo brain cells but induced an abnormal transformation of rat kidney cells with a higher efficiency than wt Ad5. Initial attempts to establish foci of hr1-transformed rat kidney cells into cell lines were unsuccessful (1). However, by passaging entire cultures containing both normal and transformed cells, Ruben *et al.* (13) were able to establish and characterize hr1-transformed rat kidney cell lines. The present study of hr1 resulted from the recent observation of Ho and Williams (personal communication) that hr1 and hr2 are cold sensitive for initiation of transformation. By using CREF cells we have been able to directly isolate hr1-transformed foci at 37°C and utilize biological and molecular methods to characterize single-cell transformed clones. This manuscript reports that hr1 transforms CREF cells at 37°C with a higher frequency than wt Ad5 and that hr1 is cold sensitive for the establishment as well as for the maintenance of the transformed phenotype in CREF cells although DNA integration, transcription, and mRNA production are unchanged after a shift of transformed cells to the nonpermissive temperature.

MATERIALS AND METHODS

Cell Cultures. CREF cells (9) were grown in Dulbecco's modified Eagle's medium (DME medium) containing 5% fetal bovine serum, and 293 cells (14) were grown in DME medium containing 10% bovine serum. Both stock cultures were maintained at 37°C in a 5% CO_2/95% air humidified incubator.

Viruses. wt Ad5 was propagated and assayed in cultures of KB cells at 37°C as described (15). hr1 was a gift from Jim Williams and was propagated and assayed in 293 cells (10).

Viral Transformation Assays. Transformation assays were performed as described with slight modifications (8, 9). CREF cells were seeded at 8×10^5 cells per 5-cm plate in DME medium with 5% fetal bovine serum at 37°C, and 24 hr later cells were infected with 10 plaque-forming units/cell of wt Ad5 or

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: m.u., map unit(s); wt, wild-type; DME medium, Dulbecco's modified Eagle's medium.

hr1. After viral adsorption for 2 hr, cells were resuspended and seeded at 10^5 cells per 5-cm plate at 37°C or 32°C. Seventy-two or 96 hr after viral infection and replating, cultures were shifted to DME medium containing decreased (0.1 mM) Ca^{2+} with 5% fetal bovine serum (low Ca^{2+} medium). Cells were refed with low Ca^{2+} medium twice a week and transformed foci were scored after 6 wk. At various times between 24 hr and 3 wk after infection, mock-infected and virus-infected cultures were shifted from 37°C to 32°C or vice versa, and all plates were fixed and stained 6 wk after infection and replating.

Cloning of hr1-Transformed Cells and Studies of Their Growth Characteristics. Two transformed foci were isolated and then cloned by seeding 50 or 100 cells per 60-mm plate. Well-separated clones were isolated by using the steel cloning cylinder technique (16). The two clones (A2 and B3) were grown into cell lines and compared at 37°C and 32°C with the parental CREF cells and a wt Ad5-transformed, cloned CREF cell line (wt-3A) with respect to their saturation density in low Ca^{2+} medium and cloning efficiency in agar. The techniques used to evaluate these parameters have been described (17).

DNA Isolation and Transfer Hybridization After Agarose Gel Electrophoresis. High molecular weight DNA was extracted from transformed cells as described (18). Ten micrograms of transformed cell DNA was digested with 25 units of appropriate restriction endonuclease (New England BioLabs), and the digests were fractionated by electrophoresis in 0.6% agarose gels in 90 mM Tris·HCl/90 mM boric acid/2.5 mM Na_2EDTA. The DNA fragments then were transferred from the gels to nitrocellulose sheets (19, 20) and were hybridized to an adenovirus, nick-translated, ^{32}P-labeled probe as described (21).

RNA Analyses. Poly(A)$^+$ cytoplasmic RNA was isolated from approximately 10^7 clone A2 and B3 cells and 293 cells as described (22, 23). Virus-specific poly(A)$^+$ species were identified by fractionating denatured RNAs (5 μg) on agarose/formaldehyde gels (24) and transferring the RNAs to nitrocellulose filters (25), followed by hybridization with nick-translated, ^{32}P-labeled (21) adenovirus E1a- (0–4.5 m.u.) or E1b- (4.5–11.0 m.u.) specific DNA probes.

Total RNA from monolayers of clones A2 and B3 and 293 cells was labeled for 12 hr with 5 mCi (1 Ci = 3.7×10^{10} Bq) of [^{32}P]orthophosphate (Amersham) in medium containing one-tenth the normal concentration of phosphate, supplemented with 10% dialyzed calf serum. Cytoplasmic and nuclear RNAs were isolated and hybridized to nitrocellulose filters containing adenovirus E1a- or E1b-specific DNA fragments as described (26).

Immunofluorescent Staining. hr1-transformed CREF clones A2 and B3 and 293 cells were grown on 1.5-cm glass coverslips, fixed with acetone, treated with antisera from hamsters bearing tumors induced by AD2$^+$ND4-transformed cells, and stained with fluorescein isothiocyanate-conjugated rabbit anti-IgG. After counterstaining with rhodamine, stained cells were visualized by using an Olympus microscope with an ultraviolet light source.

RESULTS

Effect of Temperature on Morphological Transformation of CREF Cells by hr1. Infection of CREF cells with the Ad5 host range mutant hr1, followed by replating of the infected cells at 32°C and growth for 6 wk at 32°C, resulted in the absence of discrete, morphologically transformed foci (Fig. 1 and Table 1). However, if CREF cells were infected with hr1 and were grown for 6 wk at 37°C, a 5-fold higher frequency of transformation was observed in comparison with wt Ad5-infected cultures (Table 1). The cells in hr1-transformed foci were morphologically fibroblastic, whereas wt Ad5-transformed foci usually contained epithelioid cells. When hr1-infected cultures were shifted from 32°C to 37°C 24 hr–2 wk after infection, the frequency of transformation was similar to that observed in cultures grown continuously at 37°C (Table 1). However, a reduction by approximately 50% in transformation was observed if 32°C cultures were shifted to 37°C 3 wk postinfection, probably because 6 wk were

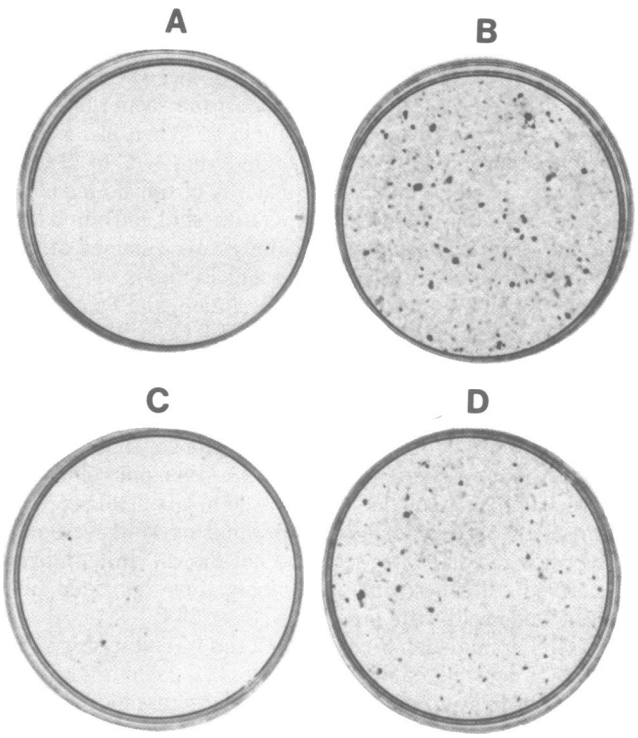

FIG. 1. Effect of temperature on transformation of CREF cells by the host range type 5 adenovirus mutant hr1. CREF cells were infected with 10 plaque-forming units/cell of hr1 and reseeded at 10^5 cells per 60-mm plate. Cultures then were incubated at: (*A*) 32°C for 6 wk; (*B*) 37°C for 6 wk; (*C*) 37°C for 2 wk and then 32°C for 4 wk; (*D*) 32°C for 3 wk and then 37°C for 3 wk.

Table 1. Effect of temperature on transformation of CREF cells by wt Ad5 and hr1 viruses

Experimental condition, °C*	Incubation period, wk	Transformation: Foci per plate, no.†	Transformation: Frequency
wt Ad5			
32	6	17 ± 4	1.7×10^{-4}
37	6	21 ± 5	2.1×10^{-4}
hr1			
32	6	0	$<1 \times 10^{-5}$
37	6	106 ± 9	1.1×10^{-3}
32 → 37	1 + 5	134 ± 11	1.3×10^{-3}
32 → 37	2 + 4	110 ± 8	1.1×10^{-3}
32 → 37	3 + 3	56 ± 7	5.6×10^{-4}
37 → 32	1 + 5	0	$<1 \times 10^{-5}$
37 → 32	2 + 4	4 ± 1	4×10^{-5}
37 → 32	3 + 3	18 ± 4	1.8×10^{-4}

* CREF cells were infected with 10 plaque-forming units/cell of wt Ad5 or hr1 and after 2 hr of viral adsorption at 37°C cells were resuspended and reseeded at 10^5 cells per 60-mm plate at 37°C or 32°C. Cultures then either were maintained continuously for 6 wk at the indicated temperature or were shifted from 37°C to 32°C or vice versa 1, 2, or 3 wk postinfection. All cultures were fixed and stained 6 wk after viral infection.

† Foci per plate is the mean number of transformed colonies or foci ± SD per 10^5 cells.

required for the maximal number of transformed foci to appear. If the reverse temperature shift was made—i.e., cultures were shifted from 37°C to 32°C—the number of foci was decreased and the degree of reduction was greatest the sooner after infection that cultures were shifted (Table 1). When plates containing hr1-transformed foci were shifted from 37°C to 32°C, 2 or 3 wk after infection at 37°C the majority of transformed foci flattened by 4–7 days after the temperature shift and only a few small foci were detectable when cultures were stained at the completion of the 6-wk assay (Fig. 1 and Table 1).

Viral DNA Integration, RNA Transcription, and Tumor Antigen Expression in hr1-Transformed CREF Cells. The pattern of viral DNA integration in chromosomal DNA of two hr1-transformed CREF cell lines grown for 2 wk at 37°C or 32°C was studied by cleavage with *Xba* I or *Hin*dIII followed by DNA transfer hybridization analysis (19). Cloned cell line A2 appeared to contain a complete copy of the Ad5 genome colinearly integrated, whereas cell line B3 appeared to be missing certain regions of the Ad5 genome (Fig. 2). Similar results were obtained when *Hin*dIII was used (data not shown). In both hr1-transformed cell lines viral DNA integration remained unchanged when cells were grown at 37°C or 32°C.

Viral RNA production in cell lines A2 and B3 cultured at 32°C or 37°C was determined by RNA transfer (24, 25) hybridization and filter (26) hybridization analysis. Total cytoplasmic poly(A)$^+$ RNA extracted from A2 or B3 cells contained three messages

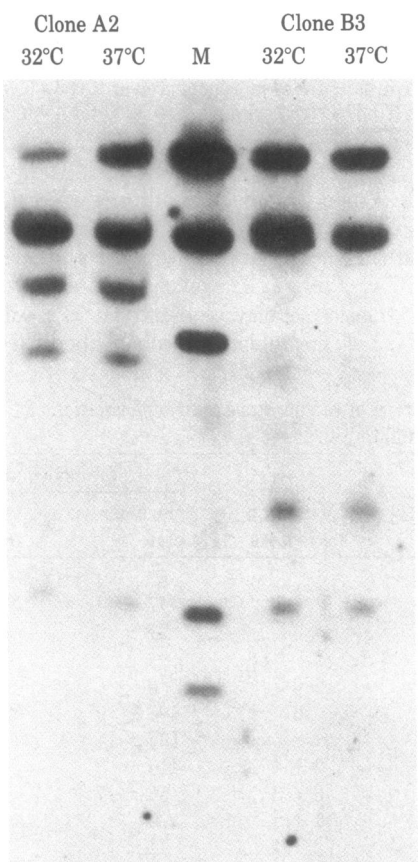

FIG. 2. Analysis of *Xba* I digests of DNA from two cloned hr1-transformed cell lines. A2 and B3 were grown for 2 wk at 37°C or 32°C prior to DNA extraction. Ten micrograms of cellular DNA was cleaved with *Xba* I and the fragments were separated by electrophoresis in 0.6% agarose, transferred to nitrocellulose sheets by blotting, and hybridized to Ad5 DNA labeled with ^{32}P by nick-translation (18, 19, 21). Cellular DNA sequences hybridizing to Ad5 DNA were detected by autoradiography. Lane M contains 10 μg from uninfected CREF cells and 5 genome equivalents of Ad5 DNA per diploid cell genome.

Table 2. RNA transcribed from E1a and E1b regions in 293 and hr1-transformed cells at 32°C and 37°C

Cell line	Temperature, °C	Total RNA, counts*			
		Cytoplasmic		Nuclear	
		E1a	E1b	E1a	E1b
hr1 A2	32	2,646	3,474	3,257	3,774
hr1 A2	37	2,428	3,915	3,362	3,483
hr1 B3	32	2,659	3,321	3,890	3,960
hr1 B3	37	2,445	3,872	4,105	4,185
293	32	4,646	4,535	3,880	3,872

* RNA was extracted from transformed cells labeled for 12 hr with [^{32}P]orthophosphate. Labeled RNA was separated into cytoplasmic and nuclear species and was hybridized to Ad2 E1a (0–4.5 m.u.) or E1b (4.5–11.0 m.u.) DNA filters. All counts shown are total counts hybridized, which have been corrected for background, determined by hybridizing labeled RNA to filters containing pBR322 DNA and, for efficiency of hybridization, determined with [^{3}H]thymidine-labeled Ad2 DNA.

of 13 S, 12 S, and 9 S, which hybridized to an E1a (0–4.5 m.u.) Ad5 probe, and two messages of 23 S and 9 S, which hybridized to an E1b (4.5–8 m.u.) Ad5 probe (data not shown). The species of RNA produced in the hr1-transformed cells were identical in cells grown at 32°C or 37°C. Filter hybridization studies, performed by immobilizing a 0- to 4.5- or 4.5- to 11-m.u. Ad5 DNA fragment on nitrocellulose filters and hybridizing with ^{32}P-labeled cytoplasmic or nuclear RNA, indicated that transcription again was similar in A2 and B3 cells cultured at 32°C or 37°C (Table 2).

Indirect immunofluorescence with immune serum from hamsters bearing tumors induced by Ad2ND4-transformed cells was used to determine if differences were apparent when A2 and B3 cells were grown at 37°C or 32°C. hr1-transformed cells grown continuously at 37°C contained intense nuclear particulate staining, whereas the same cells cultured continuously at 32°C had decreased fluorescence. When cells were shifted from 32°C to 37°C, the strong particulate nuclear staining developed by 24–48 hr. Similarly, shifting cells from 37°C to 32°C resulted in a decreased staining by 72–96 hr (data not shown).

Expression of the Transformed Phenotype in hr1-Transformed CREF Cells. In prior studies it was shown that saturation density and cloning efficiency in agar were two phenotypic characteristics that were useful in distinguishing untrans-

Table 3. Properties of uninfected, wt Ad5-transformed and hr1-transformed CREF cells grown at various temperatures

Cell line	Saturation density, cells × 10^{-5}/cm^2*		Cloning efficiency in agar, %†	
	37°C	32°C	37°C	32°C
CREF	1.2	0.7	<0.001	<0.001
Ad5wt-3A	3.2	2.9	45 ± 2	38 ± 2
Ad5hr1-A2	3.4	1.1	69 ± 6	16 ± 3
Ad5hr1-B3	3.6	1.4	79 ± 5	14 ± 1

* Approximately 10^4 cells were seeded per 35-mm plate. Cells were refed with low Ca^{2+} medium every 3 or 4 days and when cultures reached confluency cell numbers were determined by using a model Z_f Coulter Counter. Saturation densities represent the maximal cell densities obtained in confluent cultures.

† Agar cloning efficiency was determined as follows: approximately 10^3, 10^4, or 10^5 cells in low Ca^{2+} medium were prepared in 0.4% Noble agar and were seeded at 37°C or 32°C on 0.8% agar base layers prepared in the same medium. Plates were fed once a week with 2–3 ml of 0.4% agar in low Ca^{2+} medium and colonies >0.15 mm were counted after 21 days. Each value is the mean ± SD of four plates.

formed from Ad5-transformed CREF cells (9). As summarized in Table 3, both A2 and B3 had saturation densities similar to those of wt Ad5-transformed CREF cells (wt-3A) when assayed at 37°C, but the saturation density of the hr1-transformed cloned cells was lower, when they were grown at 32°C, approaching that of untransformed CREF cells. Similarly, both A2 and B3 cells grew extremely well in agar, actually exceeding the cloning efficiency of wt-3A, when they were assayed at 37°C, whereas at 32°C the cloning efficiencies of these cells were decreased by 77% and 82%, respectively, in comparison with assays performed at 37°C (Table 3). In addition, the final sizes of colonies growing in agar were much smaller in hr1 transformants grown at 32°C as compared with the cells cultured at 37°C. This is in contrast to wt transformed cells that exhibit similar sizes at both temperatures.

DISCUSSION

Although it contains a mutation in the E1a region of the genome (1, 12), the host range mutant of Ad5, hr1, can still transform sufficiently sensitive cells, such as baby rat kidney (1) or CREF (9) cells, at a relatively high frequency. The discovery that hr1 is cold sensitive to transformation, as described in this communication, helps to identify a gene product involved in adenovirus transformation and opens an approach to the investigation of its function.

Previous studies showed that incomplete transformation of rodent cells can be effected by a fragment of DNA that only encompasses the E1a region of the genome (0–4.5 m.u.) (4–6, 27). The E1a region is the initial portion of the viral DNA to be transcribed, and from these transcripts are derived 13S, 12S, and 9S mRNAs; the latter mRNA only appears during the late phase of infection (for a review, see ref. 28). How the proteins encoded in the E1a region serve to orchestrate early transcription and produce transformation is still uncertain. By using a series of engineered mutations, it has become apparent that the gene products encoded in the 12S and 9S mRNAs are dispensable and that transversions, deletions, or insertions affecting only the 13S mRNA produce host range mutants that are defective in transformation and viral replication (29, 30).

Studies with hr1 should lead to a clearer definition of the transforming function of the M_r 51,000 protein translated from the 13S mRNA. hr1 has a single base-pair deletion of nucleotide 1,055, causing a shift in the reading frame, which in turn produces the nonsense triplet TGA 11 codons downstream at nucleotides 1,085–1,088 (12). As a consequence of the stop codon produced, a truncated protein of M_r 28,000 is made in place of the M_r 51,000 acidic protein synthesized in wt virus-infected cells (12). It should be noted that the mutation in hr1 occurs within the introns of those transcripts that are processed into 12S and 9S mRNAs so that the M_r 48,000 protein translated from the 12S mRNA in wt virus-infected and transformed cells can be made. However, despite the presence of the M_r 48,000 protein, hr1 is defective in viral replication in nonpermissive cells and produces an incomplete transformation phenotype (1, 10).

It is important to note that, according to its DNA nucleotide sequence, hr1 contains a nonsense mutation (11, 12). However, hr1 acts as a conditionally lethal, cold-sensitive mutant, which is usually attributed to a missense mutation. Thus, at 37°C hr1 transforms more CREF cells than does wt virus, whereas at 32°C foci of transformed cells do not appear; but after a shift of temperature from 32°C to 37°C transformed foci appear at the usual frequency, even if incubated at 32°C for 2 wk before the temperature shift. Moreover, foci of transformed cells flatten and the phenotypic expression of the transformed cells recedes when the temperature is shifted from 37°C to 32°C. Indeed, the alternative appearance and disappearance of transformed foci can be produced indefinitely by shifting temperature.

It may be argued that only a limited segment consisting of 138 nucleotides of the E1a region was subjected to sequence analysis (12) and that, because hr1 was produced by treatment of virus with nitrous acid, a general mutagen, a second mutation, a missense mutation, may be present. To test this argument, a mutant was constructed that contains a 5-base-pair deletion at coordinate 2.85: the resulting deletion resulted in a host range mutant which, like hr1, is cold sensitive for transformation (unpublished data). Therefore, it seems likely that the single base-pair deletion in hr1 is in fact the mutation responsible for the cold-sensitive, transformation phenotype. These data imply that the truncated M_r 28,000 protein of hr1 can still effect transformation at 37°C, but that its reaction, possibly with E1b gene products and cellular components, is ineffective at 32°C because its altered structure decreases its binding constant and therefore does not permit effective associations at the lowered temperature. It may be further concluded that the M_r 51,000 acidic protein encoded in the E1a region is essential not only to establish but also to maintain the phenotypic expression of transformation.

Note Added in Proof. A study of Ho *et al.* (31) describing the cold-sensitive transformation phenotype of hr1 has appeared.

We thank Ms. Nadirah Hameed for typing this manuscript. This work was supported by National Institute of Allergy and Infectious Diseases Grants AI-12052 and AI-12053 and National Cancer Institute Grant CA-26056.

1. Graham, F. L., Harrison, T. J. & Williams, J. F. (1978) *Virology* **86**, 10–21.
2. Solnick, D. & Anderson, M. A. (1982) *J. Virol.* **42**, 106–113.
3. Jones, N. & Shenk, T. (1979) *Cell* **17**, 683–689.
4. Graham, F. L., Abrahams, P. J., Mulder, C., Heijneker, H. L., Warnaar, S. O., DeVries, F. A. J., Fiers, W. & van der Eb, A. J. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 637–650.
5. van der Eb, A. J., Mulder, C., Graham, F. L. & Houweling, A. (1977) *Gene* **2**, 115–132.
6. Houweling, A., van den Elsen, P. J. & van der Eb, A. J. (1980) *Virology* **105**, 537–550.
7. Shiroki, K., Maruyama, K., Saito, I., Fukui, Y. & Shimojo, H. (1981) *J. Virol.* **38**, 1048–1054.
8. Fisher, P. B., Mufson, R. A., Weinstein, I. B. & Little, J. B. (1981) *Carcinogenesis* **2**, 183–187.
9. Fisher, P. B., Babiss, L. E., Weinstein, I. B. & Ginsberg, H. S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3527–3531.
10. Harrison, T., Graham, F. & Williams, J. (1977) *Virology* **77**, 319–329.
11. van Ormondt, H., Maat, J., DeWaard, A. & van der Eb, A. J. (1978) *Gene* **4**, 309–328.
12. Ricciardi, R. P., Jones, R. L., Cepko, C. L., Sharp, P. A. & Roberts, B. E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6121–6125.
13. Ruben, M., Bacchetti, S. & Graham, F. L. (1982) *J. Virol.* **41**, 674–685.
14. Graham, F. L., Smiley, J., Russell, W. C. & Nairu, R. (1977) *J. Gen. Virol.* **36**, 59–72.
15. Ensinger, M. J. & Ginsberg, H. S. (1972) *J. Virol.* **10**, 328–339.
16. Fisher, P. B., Weinstein, I. B., Eisenberg, D. & Ginsberg, H. S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2311–2314.
17. Fisher, P. B., Goldstein, N. I. & Weinstein, I. B. (1979) *Cancer Res.* **39**, 3051–3057.
18. Dorsch-Hasler, K., Fisher, P. B., Weinstein, I. B. & Ginsberg, H. S. (1980) *J. Virol.* **34**, 305–314.
19. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
20. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683–3687.
21. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
22. Esche, H., Mathews, M. & Lewis, J. B. (1980) *J. Mol. Biol.* **142**, 399–417.

23. Singer, R. H. & Penmann, S. (1973) *J. Mol. Biol.* **78**, 321–334.
24. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M. & Darnell, J. E., Jr. (1981) *Cell* **23**, 731–739.
25. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
26. Nevins, J. R. (1980) *Methods Enzymol.* **65**, 768–785.
27. Shiroki, K., Shimojo, H., Sawada, Y., Uemzu, Y. & Fujinaga, K. (1979) *Virology* **95**, 127–136.
28. Ziff, E. B. (1980) *Nature (London)* **287**, 491–499.
29. Solnick, D. & Anderson, M. (1982) *J. Virol.* **42**, 106–113.
30. Carlock, L. R. & Jones, N. C. (1981) *J. Virol.* **40**, 657–664.
31. Ho, Y., Galos, R. & Williams, J. (1982) *Virology* **122**, 109–124.