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Human Chromosome 12 Is Required for Elevated HIV-1 Expression in Human-Hamster Hybrid Cells

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Host cell factors act together with regulatory genes of the human immunodeficiency virus (HIV) to control virus production. Human–Chinese hamster ovary hybrid cell clones were used to probe for human chromosomes involved in regulating HIV gene expression. DNA transfection experiments showed that 4 of 18 clones had high levels of HIV gene expression measured by both extracellular virus production and trans-activation of the HIV long terminal repeat in the presence of the trans-activator (*tat*) gene. Karyotype analyses revealed a 94% concordance (17/18) between human chromosome 12 and HIV gene expression. Other chromosomes had an 11 to 72% concordance with virus production.

THE AMOUNT OF TRANSCRIPTION and virus production of human immunodeficiency virus type 1 (HIV-1) is controlled by trans-acting regulatory genes encoded in the viral genome (*1*) and by agents known to activate T cells (*2*). Cellular factors are involved in mediating the response to both the trans-activating genes (*3–5*) and T cell activators (*4, 6–8*). The trans-activator response element (TAR) of the viral long terminal repeat (LTR) is required for *tat*-induced trans-activation of the LTR (*9*). *Tat* has not been shown to bind directly to TAR, suggesting that cellular factors are involved. Cellular proteins can bind to TAR in the absence of the *tat* protein (*3, 10*). The relation between these DNA binding proteins associated with TAR and the regulation of *tat*-directed trans-activation is not known.

We wished to identify the human chromosomes that encode cellular factors, which, in the presence of *tat*, support enhanced HIV gene expression. A series of human–Chinese hamster ovary (CHO) hybrid cell clones that contain defined sets of human chromosomes were assayed for production of extracellular HIV and for *tat*-induced trans-activation of the LTR. The results indicate that the presence of both human chromosome 12 and the HIV *tat* gene was necessary for high levels of viral gene expression in the human-hamster hybrid cells.

The ability of the hybrid clones to produce extracellular virus was tested by transfection with an infectious molecular clone of HIV DNA, pZ6neo (*11*) (Fig. 1). The parent CHO cell line and 12 of 18 hybrid clones transfected with pZ6neo DNA did not produce detectable levels of virus 3 or 6 days after transfection. Clones 151, 271, 863, and 907 produced significant amounts of extracellular virus on day 3 and more on day 6 (Fig. 1). The amount of HIV antigen produced from the human RD cell line (20-fold above cutoff; day 6), the positive control for virus production, was always greater than that of the human-hamster hybrid clones (3.4- to 5.9-fold above cutoff; day 6). Although two hybrid clones, 488 and 671, produced some detectable extracellular virus on day 6, the amount was much lower than that in the other four virus-positive clones. Intracellular viral p24 was assayed to identify any hybrid clone that synthesized viral proteins but did not assemble and export the virus. Intracellular viral p24 levels, however, did correlate to extracellular particle-associated p24 (*12*) indicating that the rate-limiting step in virus production occurred before viral protein synthesis. Cell growth rates (*12*), DNA uptake, and the ability of the cells to express transfected DNA (Table 1) did not correlate to HIV production.

Hybrid clones with enhanced virus production (clones 151, 271, 863, and 907 were at least 28% discordant with all human chromosomes except for chromosome 12, which was 6% (1 of 18) discordant (Table 1). Without human chromosome 12, hybrid clones could not support enhanced virus production, which indicated that genes encoded on this chromosome were involved in the increase of HIV gene expression. Hybrid clones 151, 271, 863, and 907 contained chromosome 12 in at least 80% of the cells tested at three separate karyotype analyses (see Table 1). Hybrid clone 864, which contained chromosome 12 but was negative for virus production (Fig. 1 and Table 1), had a continually decreasing percentage of cells containing chromosome 12 (70 to 0%). Because hybrid clones containing chromosomes 7, 10, and 16 could not be used for concordance analysis (see Table 1), additional experiments are necessary to determine their possible role in HIV gene expression.

Because the gene for the CD4 cell surface receptor of HIV (*13*) is encoded on chromosome 12 (*14*), the hybrid clones were assayed for cell surface CD4 molecules, which could amplify a basal level of HIV production by reinfection. Cell surface CD4, detected by immunostaining and flow cytometry (*15*), was present on only one of the four hybrid clones (907), which produced a high level of virus (*12*); clones 151, 271, 863, and RD cells, which produced HIV at a level greater than or equal to clone 907, were CD4-negative. Mouse L cells, stably transfected with human CD4 DNA, will bind HIV but are not permissive to HIV infection (*16*), further indicating that cell surface CD4 expression is not sufficient for supporting HIV infection. These results suggest that cellular factors encoded on chromosome 12, other than the CD4 molecule, were responsible for the increased HIV gene expression.

Because the presence of a functioning *tat* gene is required for enhanced viral gene expression (*17*), the hybrid clones and CHO cells were tested for their ability to support *tat* trans-activation of the LTR (Fig. 2).

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Cultures transfected with a plasmid DNA construct containing the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the HIV-LTR (pLTR-CAT) or cotransfected with pLTR-CAT and a plasmid (pSV-tat) containing the HIV *tat* gene under control of the SV40 early promoter (*18*) were harvested for CAT assays 48 hours after transfection. The hybrid clones, CHO, and RD cells transfected with pLTR-CAT alone had similar basal CAT activities; an average ± SD of 1.7 ± 0.9% conversion of chloramphenicol to acetylated chloramphenicol. For each experiment the combined CHO and hybrid clones LTR-CAT values were averaged and compared to the individual LTR-CAT values of each cell line used in that experiment. The chromosome 12–containing hybrid clones, when compared to the other hybrid clones and CHO, did not demonstrate a pattern of continuously higher basal CAT activity (*12*). Cotransfection with pLTR-CAT and pSV-*tat* produced salient differences in CAT activities between the cell lines. The *tat*-induced LTR-CAT activities were high in hybrid clones 151, 863, and 907 (7- to 7.7-fold above CHO), moderate in hybrid clones 271 and 864 (1.7- to 2.3-fold above CHO), and low (less than or equal to CHO) in the other ten hybrid clones. The hybrid clones 151, 271, 863, 864, and 907, which had the highest levels of CAT activity in the presence of *tat*, contained human chromosome 12 (Table 1). A functional *tat* gene is required for enhanced virus production (*19*). Our studies suggest that a cellular factor or factors encoded on chromosome 12 contributes to efficient *tat*-directed trans-activation and enhanced virus production.

The molecular mechanism or mechanisms by which the *tat* gene trans-activates HIV

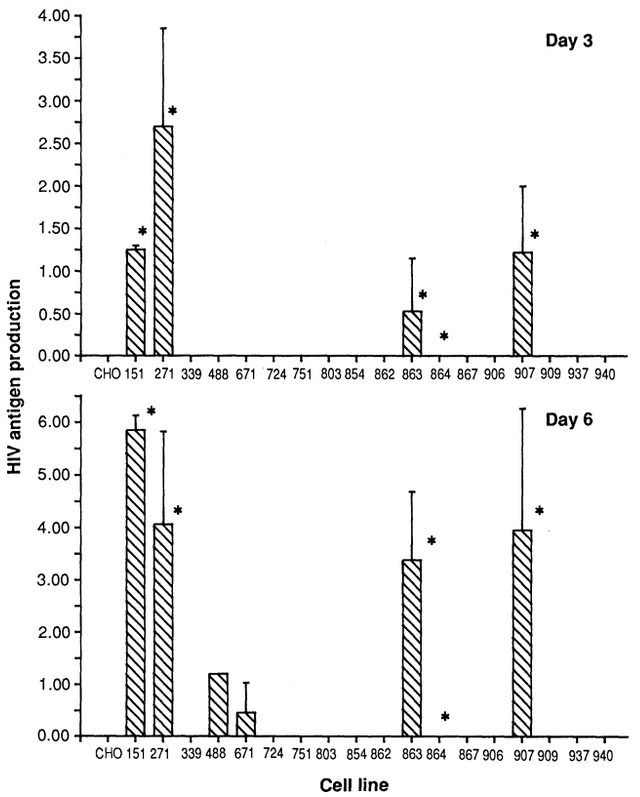

Fig. 1. HIV production in human-hamster hybrid clones. A human rhabdomyosarcoma (RD) cell line (ATCC no. CCl 136) was cultured at 37°C, in DMEM media supplemented with 10% fetal bovine serum. The parent CHO cell line, UCW 56, is a temperature-sensitive mutant grown at 34°C (*23*). Human-hamster hybrid cell clones, isolated after fusion of the parent CHO cell line and human leukocytes, were grown at the permissive temperature of 39°C (*23*). Cells (1×10^6) were transfected with plasmid DNA (10 μg) containing an infectious proviral clone of HIV (*11*) by a calcium phosphate precipitation method (*24*). Cell culture media was assayed for particle-associated HIV antigen at 3 and 6 days after transfection (*25*). The data are from seven transfection experiments; each cell line, transfected in duplicate, was used in at least two separate transfection experiments. The cutoff optical density (A_{492}) for HIV antigen production of each cell line was calculated at 0.05 above the value of a mock-transfected culture of the same cell line. The ordinate value, HIV antigen, was calculated for each cell line as the product of the average antigen value divided by the cutoff value. Error bars, SD; *, hybrid cells with chromosome 12.

Table 1. Human chromosome content of human × Chinese hamster cell hybrid clones. To evaluate the human chromosome content of the hybrid clones, karyotype analyses (*26*) were performed before, during (results of this table), and after completion of these studies. The initial series of hybrid clones used in these experiments covered all of the human chromosomes except Y.

Hybrid clone	Virus production (day 3)	Human chromosome																							DNA uptake*	pSV-CAT*
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y		
151	+												12													1.3	0.31
271	+												12													1.0	0.18
863	+	1				5	6		8				12									21	22			1.0	0.35
907	+	1			4	5							12													1.1	0.50
864	−				4	5							(12)†							19			22			1.0	0.23
339	−				(4)‡	5		(7)‡												19		21				1.0	0.35
488	−					5			8											19						ND	0.75
671	−					5	(6)‡															21		X		1.4	1.75
724	−			(3)‡		5	6					11										21				1.1	0.23
751	−					5	6							13								21				1.3	0.28
803	−				4	5																	22			1.0	0.15
854	−		2			5																				ND	0.35
862	−					5				9											(20)‡					ND	0.26
867	−	1				5								13	14				18	19						ND	0.08
906	−			3		5									14							21				1.2	0.30
909	−					5	6		8						14									X		1.1	0.16
937	−	1				5									14	15		17				21				1.3	0.13
940	−					5															20					0.9	0.13
No. concordant		13	13	12	12	2	11	§	13	13	§	13	17	12	10	13	§	13	13	10	12	9	13	12	§		
No. discordant		5	5	5	5	16	6		5	5		5	1	6	8	5		5	5	8	5	9	5	6			

*Cell cultures were assayed for DNA uptake and expression of transfected DNA (*27*) and expressed as a ratio of the values for CHO cells (=1). †Chromosome present in >30% but <70% of the cells. ‡Chromosome present in <30% of the cells. §These chromosomes were contained in hybrid clones that were excluded from analysis because of their low levels of gene expression (15-fold less than CHO) of a transfected control plasmid DNA (pSV-CAT) or the chromosome was present in less than 30% of the cells.

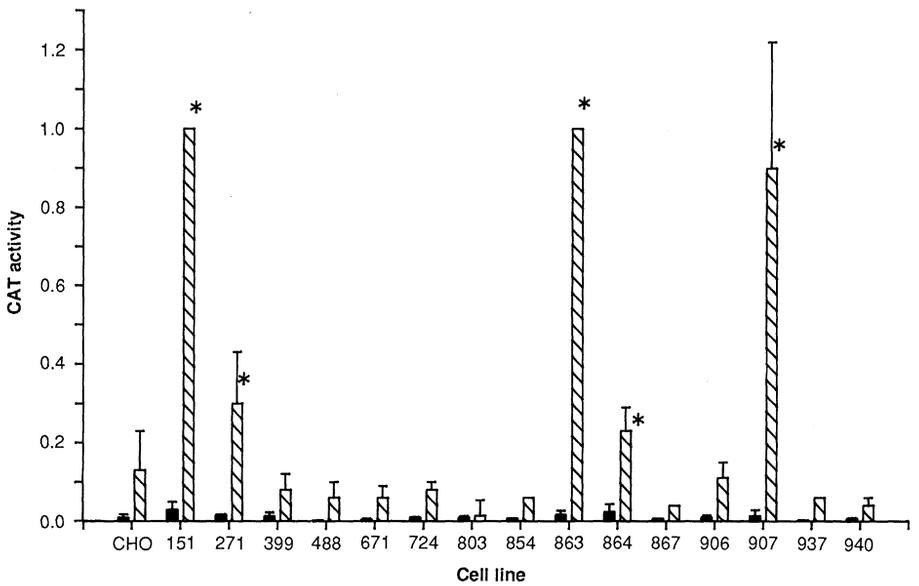

Fig. 2. *Tat* trans-activation of the HIV-LTR. Cell cultures (1×10^6) plated 18 hours before DNA transfection (*24*) with pLTR-CAT (5 μg) or pLTR-CAT (5 μg) plus pSV-*tat* (10 μg) were harvested 48 hours after transfection for CAT assays (*28*). Percent conversion of [^{14}C]chloramphenicol to acetylated [^{14}C]chloramphenicol was determined by scintillation counting of the radioactive spots from thin-layer chromatograms localized by autoradiography. For quantitation of CAT activity, conversion of chloramphenicol to its acetylated forms was measured in the linear range of the assay. To control for differences in the ability of cells to express transfected DNA, CAT values were normalized to CAT activities of the cell lines transfected with pSV-CAT (see Table 1). The values presented here are an average of at least two separate transfection experiments per cell line with duplicate plates per experiment. The ordinate, CAT activity, is the CAT activity related to the activity of hybrid clone 863 set at 1. Error bars, SD; *, hybrid cells with chromosome 12.

gene expression are not fully understood. Mutational analysis of the LTR shows that TAR is required for *tat* trans-activation of gene expression (*9*). No direct binding of the *tat* protein to TAR or to other LTR sequences has been reported. The binding of cellular proteins to TAR (*3, 10*) and the LTR enhancer region (*7, 8, 10, 20*) suggests that increases in viral gene expression in the presence of the *tat* protein may be mediated through cellular proteins. Cellular proteins isolated from cells not expressing the *tat* gene bind to sequences in the TAR region (*3*). The association between these TAR-binding proteins and chromosome 12 and the role these proteins may have in *tat* trans-activation are currently under investigation.

Cellular factors, mediated by viral trans-activating proteins, have been shown to regulate gene expression from the LTR of the human retrovirus human T cell lymphotropic virus type I (HTLV-I) (*21*). The search for cellular mechanisms involved in *tat* trans-activation of the HIV LTR has also focused on searching for factors that increase viral gene expression. Recent evidence, however, indicates that cellular factors may negatively regulate viral expression (*6, 22*). It is not known whether cellular factors encoded on chromosome 12, which we propose act in concert with *tat* to increase viral gene expression, activate a positive control mechanism or inactivate a negative control mechanism of HIV gene expression. The control of gene expression, regulated through TAR, of the human retroviruses HTLV-I and -II and HIV-1 and -2 may require similar or different cellular factors. The use of human-hamster hybrid cells containing defined sets of human chromosomes should provide a means to further identify the host cell factors, and their chromosomal location, that are involved in regulating retroviral gene expression.

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24. A modification of the $CaPO_4$ precipitation method of F. L. Graham and A. J. Van der Eb [*Virology* **52**, 456 (1973)] was used to introduce plasmid DNA into cells. Cells (1×10^6 cells per 100 mm^2) were plated 18 hours before transfection. The DNA-$CaPO_4$ precipitate was incubated with the cells for 4 to 6 hours followed by a 90-s exposure to a shock buffer containing phosphate-buffered saline (PBS), 15% glycerol, dextrose (2 g/liter), and 10 m*M* Hepes (*p*H 7.1). The cultures were immediately rinsed with the shock buffer minus glycerol, and DMEM supplemented with 10% fetal bovine serum was added to the cultures.
25. Antigen assay for virus production was done as follows. Media from cell cultures transfected with pZ6neo DNA were monitored for particle-associated HIV p24 with an enzyme immunoassay (Abbott Laboratories, North Chicago, IL) (*30*). Cells were removed by centrifugation, and the supernatant centrifuged 100,000*g* for 1.5 hours. The resulting pellet was treated with solubilization buffer (200 μl) that contained 50 m*M* tris-HCl (*p*H 7.8), 20% glycerol, 0.8*M* NaCl, 0.5% Triton X-100, 5 m*M* EDTA, and 1.0 m*M* phenylmethylsulfonyl fluoride (PMSF). Bovine serum albumin was added to the solubilized pellet to a final concentration of 1%. Intracellular HIV p24 was also measured by the same assay with the following modifications. Ice-cold PBS that contained 2 m*M* EDTA was added to the cultures for 10 min. Adherent cells were dislodged from the culture dish by gentle tapping. The cells were pelleted, washed with cold PBS, and treated with solubilization buffer (1 ml). The solubilized cell preparation was centrifuged to pellet high molecular weight DNA, and the supernatant was analyzed according to the manufacturer's protocol.
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27. Duplicate cell cultures (1×10^6) were transfected (*24*) with a plasmid DNA construct (pSV-CAT) containing the bacterial CAT gene driven by the SV40 early promoter (*18, 28*) and harvested 18 hours later by the Hirt procedure [B. Hirt, *J. Mol. Biol.* **26**, 365 (1967)]. The episomal DNA fraction was dot blotted to nitrocellulose and probed with ^{32}P-labeled nick-translated pSV-CAT. Episomal DNA fractions from mock-transfected cultures served as controls. Autoradiograms taken from the dot blot analysis were quantitated with a video densitometer (Bio-Rad Laboratories, Richmond, CA). DNA uptake of the hybrid clones was calculated as a ratio of CHO DNA uptake. CAT activity (*28*) was measured 48 hours after transfection of duplicate cultures with pSV-CAT (10 μg). Hybrid clones CAT activity was calculated as a ratio of the CHO CAT activity. DNA uptake and CAT activity did not vary more than 20% between duplicate cultures of the same cell line.
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30. Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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p53: A Frequent Target for Genetic Abnormalities in Lung Cancer

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Allele loss is a hallmark of chromosome regions harboring recessive oncogenes. Lung cancer frequently demonstrates loss of heterozygosity on 17p. Recent evidence suggests that the p53 gene located on 17p13 has many features of such an anti-oncogene. The p53 gene was frequently mutated or inactivated in all types of human lung cancer. The genetic abnormalities of p53 include gross changes such as homozygous deletions and abnormally sized messenger RNAs along with a variety of point or small mutations, which map to the p53 open reading frame and change amino acid sequence in a region highly conserved between mouse and man. In addition, very low or absent expression of p53 messenger RNA in lung cancer cell lines compared to normal lung was seen. These findings, coupled with the previous demonstration of 17p allele loss in lung cancer, strongly implicate p53 as an anti-oncogene whose disruption is involved in the pathogenesis of human lung cancer.

SPECIFIC CHROMOSOMAL DELETIONS have been reported in various human tumors, suggesting that anti-oncogenes ("tumor suppressor" genes) are important in the pathogenesis of these malignancies (*1*). Lung cancer cells appear to have many such abnormalities. Besides exhibiting many structural and numerical cytogenetic changes (*2*), comparison of tumor and normal tissue DNAs by means of restriction fragment length polymorphism (RFLP) probes revealed loss of heterozygosity in chromosome regions 3p, 13q, and 17p (*3–5*). Allele loss is highly suggestive of the presence of an anti-oncogene (*1*) and loss of the 13q allele provided a signpost leading to the discovery of inactivation of the retinoblastoma gene (*Rb*) in many, if not all, small cell lung cancers (SCLC) (*6*). Similarly, allele loss for chromosome regions 3p and 17p in both SCLC and non–small cell lung cancer (non-SCLC) suggested a search for anti-oncogenes in these chromosome areas. Since p53 is assigned to chromosome region 17p13 (*7*) and recent in vitro studies have suggested the possibility that p53 acts as an anti-oncogene (*8–10*), we explored the status of the p53 gene in lung cancer.

We examined 30 lung cancer cell lines (*11*) (13 SCLC, 14 non-SCLC, 2 extrapulmonary small cell carcinomas, and 1 pulmonary carcinoid) as well as samples from normal lung obtained at the time of surgical resection for lung cancer for p53 abnormalities. We used Southern (DNA) and Northern (RNA) blot analyses with a probe isolated from a normal human p53 cDNA clone (*12*) (Fig. 1 and Table 1). Two of the lung cancer cell lines (H358 and H660) showed homozygous deletions, one (H969) exhibited a genomic p53 DNA rearrangement, and the remainder showed no gross DNA structural abnormalities. H358 and H969 expressed no detectable mRNA even by the ribonuclease (RNase) protection assay (see below), whereas H660 exhibited very low level expression of a truncated 1.4-kb p53 mRNA. Four lines (H526, H82, H676, and H647) without gross structural DNA abnormalities expressed varying levels of abnormally sized mRNAs (2.6, 3.7, 2.3, and 3.7 kb, respectively), suggesting abnormal splicing, initiation, or termination. H676 also expressed a normally sized (2.8-kb) p53 mRNA as well as an abnormally sized one. Others expressed normally sized p53 mRNA at similar levels (for example, H23) or reduced levels (for example, H889) compared to normal lung.

After discovering these gross abnormalities in the p53 gene, we wished to know if lung cancer cells expressing apparently normally sized transcripts contained subtle mutations and whether these occurred in tumor specimens taken directly from patients without intervening cell culture. We therefore performed RNase protection assays (*13*) using three overlapping probes isolated from a normal human p53 cDNA clone, which together covered nearly the entire coding region of the human p53 gene (*12*) (Fig. 2). RNAs from eight normal lung samples showed only full-length protection with the p53M and p53PA probes (*14*). In contrast, a SCLC (T1436) and a non-SCLC (T104) tumor sample showed abnormal RNase

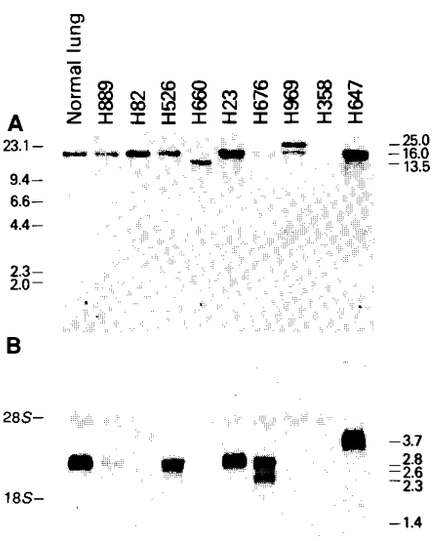

Fig. 1. The p53 DNA status and mRNA expression in lung cancer cell lines and normal human lung. (**A**) Southern blot analysis of p53 Eco RI–digested lung cancer cell line and normal lung DNAs (10 μg per lane). (**B**) Northern blot analysis of p53 in total cellular RNA (10 μg per lane) from lung cancer cell lines and a representative example of normal lung. In (A) numbers on the left indicate marker sizes in kilobases and numbers on the right indicate normal (16 kb) and abnormally sized p53 Eco RI fragments (in kilobases). Ethidium bromide staining showed amount of DNA loaded per lane is approximately equal except H676, which had less loaded than others. In (B) numbers on the right show both normal (2.8 kb) and abnormal p53 mRNA sizes (in kilobases), whereas the 28*S* ribosomal band serves as an internal marker for amount of RNA loaded. The probe used is a 1.8-kb Xba I–Xba I fragment prepared from a normal human p53 cDNA clone, php53c1, labeled with ^{32}P by the random primer technqiue (*26*). Methods for preparing DNA, RNA, and probe fragments along with analysis of Southern and Northern blots were as previously described (*27*). Additional restriction digests with Hind III and Bam HI confirmed the DNA abnormalities found in H358, H969, and H660 with Eco RI (*14*).

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