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15. Although mtlrRNA is present in mitochondria [M. Binder *et al.*, *J. Cell Biol.* **102**, 1646 (1986)], we could not detect an mtlrRNA signal in mitochondria by our in situ hybridization technique because we sectioned embryos for electron microscopy after in situ hybridization (*18*). This is probably because the mtlrRNA probe hardly permeates mitochondrial membrane under the conditions used.
16. In situ hybridization to whole-mount Oregon-R embryos was carried out principally according to Tautz and Pfeifle (*27*). We used a double-stranded DNA probe for in situ hybridization.
17. Sense and antisense DNA probes were obtained by asymmetric polymerase chain reaction with a full-length complementary DNA (cDNA) of mtlrRNA (*28*) as a template.
18. In situ hybridization of the mtlrRNA cDNA probe to whole-mount Oregon-R embryos was carried out principally according to Tautz and Pfeifle (*27*), but we used antibody to digoxigenin (anti-digoxigenin) (Bio Cell Research Laboratories, Cardiff, U.K.) that was conjugated to 1-nm gold particles instead of alkaline-phosphatase–conjugated anti-digoxigenin. After the antibody reaction, the immunogold-labeled embryos were processed for silver enhancement with a silver enhancing kit (Bio Cell). It is well known that gold catalyzes the reduction of silver ions to metallic silver. Thus, in preparations processed for silver enhancement, gold particles are surrounded by a growing shell of metallic silver [G. Danscher, *Histochemistry* **71**, 81 (1981)]. In embryos hybridized with an antisense mtlrRNA probe and processed as above, a signal localized in the polar plasm was easily observed under a light microscope (Fig. 2B, inset). The embryos were embedded in epoxy resin for transmission electron microscopy. Thin sections were cut with an LKB Nova Ultramicrotome (LKB, Bromma, Sweden) and observed under a JEM 100CXII electron microscope (JEOL, Tokyo, Japan). The detailed method for in situ hybridization for electron microscopy can be found elsewhere (R. Amikura *et al.*, in preparation).
19. We counted the number of signals in areas that were a total of 500 μm^2 in the polar plasm and in the same areas in the lateral region of early cleavage embryos (within 60 min after egg laying). In embryos hybridized with an antisense probe for mtlrRNA, we observed 167 signals in the polar plasm and 63 in the lateral region. One hundred twenty-two signals (73% of the total signals) in the polar plasm were present on the surface of polar granules. The remaining signals in the polar plasm and all signals in the lateral region were scattered all over the cytoplasm, including the mitochondria. These observations show that enrichment of the signal in the polar plasm is not a result of an intramitochondrial signal but a result of an extramitochondrial signal on polar granules. In the control embryos hybridized with a sense probe, we observed 59 signals in the polar plasm and 32 in the lateral region. No signal was present on the polar granules, and all signals were on mitochondria or in residual cytoplasm. These observations suggest that almost all signals on mitochondria and in the residual cytoplasm represent background.
20. As shown in Fig. 2, the mtlrRNA signal can be observed on one side of the polar granules. The side with the signal may be the side previously associated with mitochondria because the mtlrRNA signal is frequently found at the boundaries between mitochondria and polar granules in very early cleavage embryos (within 30 min after egg laying). In 250-μm^2 areas in the polar plasm of a very early cleavage embryo, we found 33 signals on polar granules associated with mitochondria and 32 on free polar granules. Of the 33 signals on polar granules associated with mitochondria, 30 signals were observed at the boundaries between polar granules and mitochondria (Fig. 2H).
21. In situ hybridization was carried out in the presence of 300 times excess (relative to the labeled antisense mtlrRNA probe) of an unlabeled antisense RNA that was transcribed from a full-length cDNA of mtlrRNA in vitro.
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30. In situ hybridization was carried out as described (*27*). Embryos (*mwh* e^{11}) were UV-irradiated as described [S. Togashi, S. Kobayashi, M. Okada, *Dev. Biol.* **118**, 352 (1986)]. The genotype of the females that produced *Bic-D* embryos was *b dp Bic-D*$^{71\ 34}$*/CyO*.
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Predisposition to Neoplastic Transformation Caused by Gene Replacement of H-*ras*1

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Homologous recombination was used to introduce a nominally transforming mutation into an endogenous H-*ras*1 gene in Rat1 fibroblasts. Although both the mutant and the remaining normal allele were expressed equally, the heterozygous cells were not neoplastically transformed. Instead, spontaneously transformed cells arose from the heterozygotes at a low frequency, and the majority of these cells had amplified the mutant allele. Thus, the activated H-*ras*1 allele was not by itself dominant over the normal allele but predisposed cells to transformation by independent events, such as amplification of the mutant allele.

Certain point mutations within coding sequences of *ras* proto-oncogenes (H-*ras*, K-*ras*, and N-*ras*) generate oncogenes that, when ectopically expressed, can transform rodent cell lines (*1*). Furthermore, the transforming ability of these mutant alleles prevails even though the normal alleles are also expressed (*1*, *2*). The mutant alleles have therefore been referred to as dominant. Consistent with this assessment, the mutant genes are gain-of-function alleles; in general, their protein products have lost the ability to hydrolyze guanosine triphosphate efficiently and, thus, become constitutively activated (*1*, *3*). Although the mutant *ras* alleles are strongly implicated in human tumorigenesis (*1*, *4*, *5*), their proposed dominance has been questioned (*6*).

To address the issue of *ras* dominance, we used homologous recombination to replace one copy of normal H-*ras*1 in Rat1 fibroblasts [subclone B1a(+/+) (*7*)] with the mutant allele NMU-H-*ras*1 and then assessed whether the heterozygous cells were neoplastically transformed. The NMU-H-*ras*1 gene (isolated from rat mammary tumors after mutagenesis with nitrosomethylurea) contains a single missense mutation that converts Gly^{12} to Glu (*8*) and can transform Rat1 fibroblasts when expressed in abundance after DNA transfection (*8*, *9*).

We replaced H-*ras*1 with NMU-H-*ras*1 by a two-step method that resembles strategies used previously in yeast (*10*) and mammalian cells (*11*). The first step required integration of the vector DNA that contained a truncated mutant *ras* allele into the chromosomal H-*ras*1 locus by homologous recombination (Fig. 1). The resultant cells were neoplastically transformed because the recombination event reconstituted a full-length NMU-H-*ras*1 allele expressed from the strong promoter of the murine leukemia virus–long terminal repeat (MLV-LTR). These transformed cells, referred to as Me12/+, also possessed a truncated normal H-*ras*1 allele and a copy of the *gpt* gene driven by the SV40 promoter-enhancer (SV-*gpt*) that rendered them resistant to mycophenolic acid.

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The second step in the gene replacement strategy required excision of the integrated vector DNA by intramolecular reciprocal exchange between the truncated H-*ras*1 gene and the full-length, mutant allele (Fig. 1). Cells that had excised DNA including the *gpt* gene were selected by resistance to 6-thioguanine (6-TG). When the exchange occurred 5′ to codon 12, the resultant cells retained a full-length mutant *ras* allele that was expressed from its own natural promoter rather than from the MLV-LTR. These heterozygous cells, referred to as e12/+, were then characterized in detail.

The relative expression of normal and mutant H-*ras*1 RNA in B1a(+/+), Me12/+, and e12/+ cells was assessed. Total H-*ras*1 expression in Me12/+ cells was ten times greater than that in the parental B1a(+/+) cells (Fig. 2A), and the majority of this RNA contained the C-to-T mutation at nucleotide 35 (the nucleotide responsible for the codon 12 mutation) (Fig. 2B). In contrast, total H-*ras*1 expression in e12/+ cells was similar to that in B1a(+/+) cells (Fig. 2A), and about half of this RNA contained the C-to-T mutation (Fig. 2B). We therefore conclude that both the normal and mutant alleles of H-*ras*1 were expressed in normal amounts in e12/+ cells.

To determine whether mutant Ras protein was expressed in the various cells, we performed immunoprecipitations with antibodies Y13-238 and Y13-259, which recognize all forms of Ras, and then immunoblotted them with pan-Ras Glu^{12}, an antibody specific for mutant Ras. The mutant protein was readily detected in both e12/+ and Me12/+ cells but not in B1a(+/+) cells (Fig. 2C). The amount expressed in Me12/+ cells was about 20 times greater than that in e12/+ cells, most likely because of the strong MLV-LTR promoter. Normal Ras was readily detected in B1a(+/+) cells when the immunoblot was stripped of antibody and reprobed with pan ras (Ab2), an antibody that recognizes both mutant and normal Ras. (Fig. 2D).

To test whether the mutant allele in e12/+ cells was dominant over the normal allele, we analyzed the cells for morphological transformation, disturbance of actin cables, growth in suspension, and tumorigenesis in nude mice. The parental B1a(+/+) and the e12/+ cells were not morphologically transformed; the cells were flat and nonrefractile and grew as a monolayer, and they exhibited normal actin cables (Fig. 2E). In contrast, Me12/+ cells were morphologically transformed; the cells were highly elongated and refractile, did not grow as a monolayer, and did not exhibit actin cables (Fig. 2E). To test the ability of cells to grow in suspension (*12*), we plated B1a(+/+) and e12/+ cells (10^6 cells each) and 10^3 Me12/+ cells in soft agar cultures. After 18 days, 10% of the plated Me12/+ cells produced colonies. In contrast, no colonies were obtained from B1a(+/+) cells and only three colonies (a cloning efficiency of 6×10^{-6}) were obtained from e12/+ cells. Finally, to assess the tumorigenic capacity of Me12/+ and e12/+ cells, we grafted 10^6 cells of each line under the kidney capsule in nude mice (*13*). After 3 weeks, tumors were present in six out of six Me12/+ grafts but in none of six e12/+ grafts (9).

We conclude that e12/+ cells are not transformed and the mutant H-*ras*1 allele is not dominant over the normal allele. However, because the soft agar assays with e12/+ cells did occasionally produce a few colonies, in contrast to assays with B1a(+/+) cells that never produced colonies, we hypothesized that e12/+ cells may be "predisposed" to neoplastic transformation. To test this hypothesis, we measured the rate of spontaneous focus formation for both B1a(+/+) and e12/+ cells by Luria-Delbrück fluctuation analysis (*14*) (Table 1). The results indicated that e12/+ cells spontaneously produced cells capable of focus formation at a frequency between 3.4×10^{-8} and 9.5×10^{-8} per e12/+ cell per generation. In contrast, no foci were observed in B1a(+/+) cultures (a frequency less than 5.0×10^{-9} per e12/+ cell per generation). Wild-type revertants of Me12/+ cells [referred to as R1(+/+) and R2(+/+) cells, in which the intramolecular reciprocal exchange excising the *gpt* gene was 3′ to codon 12] similarly did not produce spontaneously transformed cells (Table 1). Thus, the augmented frequency of spontaneous transformation observed within the e12/+ cells was not a residual effect of the transformed phenotype of the Me12/+ cells from which they were derived.

We used restriction analysis of DNA to investigate the spontaneous transformation of the e12/+ cells (Fig. 3). Because of an Eco RI restriction site polymorphism within

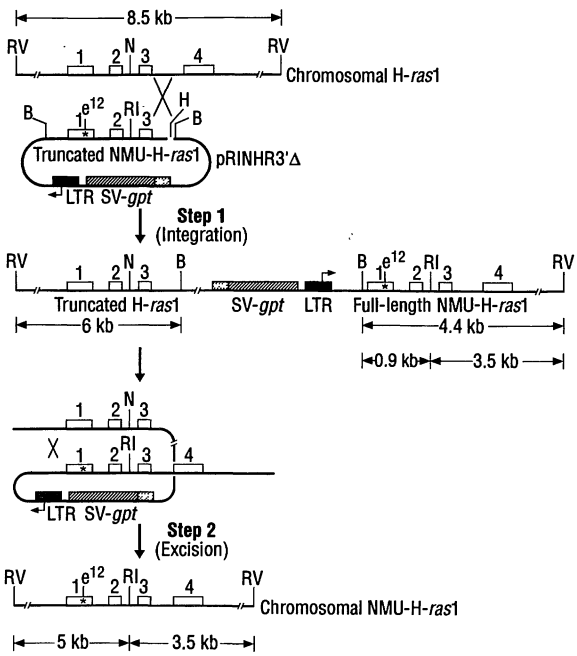

Fig. 1. Strategy for replacing H-*ras*1 with NMU-H-*ras*1. Step 1: integration of plasmid pRINHR3′Δ into a chromosomal copy of H-*ras*1 by homologous recombination (*20*). Step 2: excision of DNA by intramolecular homologous recombination 5′ to codon 12. The genotype of cloned cells was determined by restriction enzyme digestion of genomic DNA and Southern (DNA) blot analysis (*9, 21*). After step 2, DNA sequencing was used to demonstrate that the mutant allele contained the same G-to-A mutation observed in NMU-H-*ras*1 (*9*).

Table 1. Frequency of spontaneous transformation. We plated B1a(+/+), e12/+, R1(+/+), or R2(+/+) cells onto 60-mm tissue culture dishes (60 dishes per cell line, 500 cells per dish) and counted the number of cultures that did not contain foci 3 weeks after the cultures reached confluence. The average number of cells per 60-mm culture at confluence was determined with a hemocytometer. A Luria-Delbrück fluctuation analysis (*14*) was used to compute the rate of spontaneous transformation.

Cell type	Experiment	Cell divisions per culture	Cultures without foci (%)	Frequency of spontaneous transformation
B1a(+/+)	1	1.40×10^6	100	$<5.0 \times 10^{-9}$
B1a(+/+)	2	1.23×10^6	100	$<5.6 \times 10^{-9}$
e12/+	1	1.55×10^6	92	3.7×10^{-8}
e12/+	2	1.35×10^6	83	9.5×10^{-8}
e12/+	3	1.43×10^6	93	3.4×10^{-8}
R1(+/+)	1	1.40×10^6	100	$<5.0 \times 10^{-9}$
R2(+/+)	1	1.29×10^6	100	$<5.4 \times 10^{-9}$

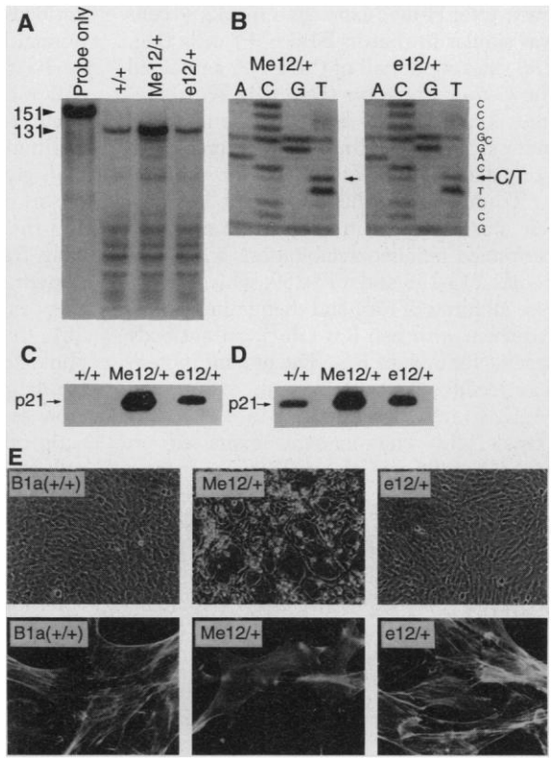

Fig. 2. Expression of and morphological features of B1a(+/+), Me12/+, and e12/+ cells. (**A**) Ribonuclease protection analysis of H-*ras*1 expression. The probe was the 151-nucleotide Sac I fragment of rat H-*ras*1 and included 131 nucleotides at the 5′ end of the first exon (the protected portion) plus 20 nucleotides of the first intron (the unprotected portion). Samples of total RNA (10 μg) from B1a(+/+) (+/+), Me12/+, and e12/+ cells were hybridized (*22*). (**B**) Relative expression of normal and mutant H-*ras*1 RNA. The polymerase chain reaction (PCR) was used to amplify H-*ras*1 sequences, including nucleotide 35 (arrow), from Me12/+ and e12/+ cells. The 5′ primer sequence GCAGGAGCTCCTGGRTTTGGC was 5′ to the initiating ATG codon. The 3′ primer sequence GACTACGTGTTTCCGGTAGGAGTC was at the 5′ end of exon 2. The 145-nucleotide PCR product was purified by agarose gel electrophoresis and then sequenced directly (U.S. Biochemical, Cleveland, Ohio). (**C**) Expression of mutant Ras protein. Ras protein from subconfluent cultures was immunoprecipitated with antibodies Y13-238 and Y13-259 and protein G–Sepharose (Zymed Laboratories, South San Francisco, California). After separation on 12.5% acrylamide gels, the immunoprecipitated protein was immunoblotted with pan-Ras Glu^{12} (Oncogene Science, Uniondale, New York) and visualized with a sheep antibody to mouse immunoglobulin G conjugated to horseradish peroxidase (Organon Teknika, Durham, North Carolina) and enhanced chemiluminescence (Amersham, Arlington Heights, Illinois) (*23*). (**D**) Expression of normal Ras protein. The immunoblot in (C) was stripped of antibodies and reprobed with pan ras (Ab2) (Oncogene Science). (**E**) B1a(+/+), Me12/+, and e12/+ cells were each grown to a density of 10^6 cells per 100-mm plate and observed by phase microscopy at ×200 magnification (top micrographs). We determined the presence of actin cables (bottom micrographs) by plating cells onto fibronectin-coated glass slides by fixation with 2% paraformaldehyde and by staining with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, Oregon).

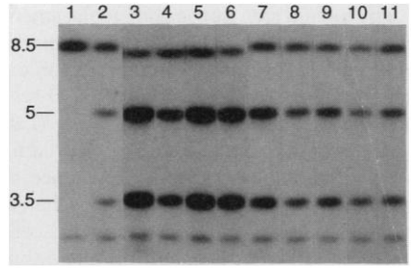

Fig. 3. Southern analysis of H-*ras*1 sequences in spontaneously transformed cell lines. Genomic DNA (15 μg) was cut with Eco RV and Eco RI and subjected to electrophoresis through 0.7% agarose gels and a Southern blot probed with a nick-translated 2-kb Bam HI fragment from pRINHR3′Δ. Lane 1, B1a(+/+); lane 2, e12/+; lane 3, A1e12/+; lane 4, A4e12/+; lane 5, A5e12/+; lane 6, A6e12/+; lane 7, A7e12/+; lane 8, A8e12/+; lane 9, A9e12/+; lane 10, A10e12/+; lane 11, A11e12/+. This figure is a composite from two gels. Molecular size markers are shown to the left in kilobases.

the second intron of the mutant H-*ras*1 allele (Fig. 1), this allele was represented by bands at 5 and 3.5 kb. The 8.5-kb band represented the normal allele. Because all spontaneously transformed cell lines examined contained the normal allele of H-*ras*1, spontaneous transformation did not correlate with the loss of the normal allele. Instead, in 14 out of 15 transformed lines, hybridization to the mutant allele of H-*ras*1 was augmented. Only one transformed cell line retained the mutant allele as a single copy (*9*). Thus, spontaneous transformation of e12/+ cells correlated with amplification of the mutant *ras* allele. The number of copies of mutant H-*ras*1 ranged from two or three in A8e12/+ and A10e12/+ cells to 11 to 12 in A5e12/+ cells (*15*).

We have directly demonstrated that a single point mutation in H-*ras*1 is not in itself sufficient for neoplastic transformation of even established cell lines; at least one additional event, such as gene amplification, is required. Because the e12/+ cells are heterozygous for mutant H-*ras*1, they are a reasonable model of cells enroute to neoplastic transformation in vivo. Consistent with this conclusion, expression of mutant *ras* genes is frequently augmented in tumor cells by gene amplification or other mechanisms (*4, 5, 16*). Even EJ H-*ras*1, the original activated *ras* allele isolated from human cells, is now known to contain not only a codon 12 mutation (*17*) but also a mutation within the last intron that augments expression of the gene over tenfold (*18*). But other independent events may also suffice. For example, loss of normal H-*ras*1 has been observed in human tumors (*4, 5*) and is a consistent feature of mouse skin tumor development (*19*), which suggests that the absence of the normal gene product may facilitate transformation by the remaining mutant product. In addition, many human tumors (*4, 5*) and at least one spontaneously transformed cell line derived from e12/+ cells (*9*) retain a single copy of mutant H-*ras*1, which indicates the importance of events unrelated to gene dosage. The e12/+ cell line described here may facilitate identification of these events.

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TECHNICAL COMMENTS

Metal-Metal Bonds in Bimetallic Surfaces

J. A. Rodriguez and D. W. Goodman show (*1*) an interesting correlation between core-electron binding-energy shifts and desorption temperatures for monolayer metal adsorbates on transition metal substrates. From the shift they deduce a charge transfer that turns out to be "completely contrary to that observed in bulk alloys." This deduction is most unexpected and warrants further discussion. We used a Born-Haber cycle to clarify the origin of the observed correlation of binding-energy shift with desorption temperature and point out that there are other important contributions to the shift, not mentioned in the article, that preclude a simple connection with charge transfer.

The correlation between binding energy and desorption temperature has a well-known theoretical basis. It arises because the desorption temperature provides a measure of the adsorption enthalpy. A simple expression for the core-electron binding-energy shift from the bulk metal to the adsorbed monolayer, each measured relative to its own Fermi level, is readily obtained from the corresponding Born-Haber cycles (*2*):

$$\Delta E_B^{ads} = (E_{ads}^Z - E_{ads}^{Z+1}) - (E_{coh}^Z - E_{coh}^{Z+1}) + \Delta E_{imp}^{Z+1\rightarrow Z} \quad (1)$$

In this formulation the E_{ads} are adsorption enthalpies, the E_{coh} are bulk cohesive energies, and the superscripts Z and Z + 1 denote the adsorbate element and the element with next high atomic number. The Z + 1 term enters the equation through an equivalent-cores argument (*2*). The cohesive energy terms are properties of the bulk metals and are independent of the substrate. The last term is the difference between the monolayer and bulk implantation energies (*2*). These are the energies obtained when a Z + 1 atom is moved from an environment of Z + 1 atoms to one of Z atoms. This term should be small, but it is important if quantitative results are required. Thermodynamically sound Born-Haber expressions of this type provide good estimates of core-electron binding energies in metals (*2*) as well as of binding-energy shifts of adsorbates (*3*). The important point is that the Z and Z + 1 adsorption enthalpies enter the equation on equal terms but with opposite signs. The simple correlations shown in the figures of the article by Rodriguez and Goodman tell only half the story. One should not conclude that such correlations are typical of all adsorbate systems, because even the sign of the shift will change if the Z + 1 atom is more strongly adsorbed than the Z atom. It would be interesting to compare the data in the article (*1*) with the predictions of Eq. 1.

Unfortunately, the Born-Haber treatment gives no clue about the charge transfer between adsorbate and substrate. However, binding-energy shifts in metals and alloys depend not only on charge transfer, but also on changes in reference level, hybridization, and final-state screening (*4*). The fact that charge transfer alone cannot explain the observations became inescapable when it was found experimentally in many noble and transition metal alloy systems that the core-electron binding-energy shift of both components has the same sign. This demonstrates that some of the other terms can be as large or larger than the charge-transfer contribution. Changes in screening and hybridization are likely to make large contributions for alloys with Ni and Pd. Changes in reference level are important when metals with different work functions are combined. Correlations between differences in work function and core-electron binding energy demonstrate the importance of the reference-level term. These additional terms are equally relevant to adsorbate layers. There is no more justification for using the core-electron binding-energy shift as a simple indicator of charge transfer in bimetallic systems than there is for using it in alloys. When the charge transfer in these systems is properly evaluated, the disagreement with the behavior of bulk alloys is likely to vanish.

There are cases in which charge transfer is the major source of core-electron binding-energy shifts (*5*), for example, in molecular systems and insulating solids. In these systems the charge transfer is between well-defined, localized electronic orbitals; while in bimetallic systems the charge flow is between delocalized conduction band states that may contain contributions from both substrate and overlayer orbitals, making it difficult even to define the charge transfer (*5*).

For the adsorbate systems discussed in the article by Rodriguez and Goodman (*1*), it would be of great interest to measure not only the core-electron binding-energy shift of the adsorbate but also the effect of the adsorbate on the surface layer of the substrate. This should present no difficulty because the signal from the first atomic layer of the substrate is readily resolved in photoemission (*6*) for some of the metals discussed in the article. Such data could add an important new dimension to this study and might lead to a better understanding of the interaction between substrate and adsorbate.

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Response: The conclusions in our article were based not only on a correlation between core-level binding-energy (CLBE) shifts and desorption temperatures of metal adsorbates but also on correlations between CLBE shifts and results of work function measurements, ultraviolet photoemission spectroscopy (UPS), CO thermal desorption mass spectroscopy (CO-TDS), CO Fourier transform infrared spectroscopy (CO-FTIR), and CO high-resolution electron energy loss spectroscopy (CO-HREELS). As pointed out by Wertheim and Rowe, CLBE shifts may "depend not only on charge transfer, but also on changes in reference level, hybridization, and final-state screening. . . ." Taking this into consideration, we compared the charge-transfer