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On the mechanism of nucleolar dominance in mouse–human somatic cell hybrids

(ribosomal genes/transcription factors/RNA polymerase I promoters)

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ABSTRACT **The mechanism of nucleolar dominance was studied in two lines of mouse–human somatic hybrids. Both lines had preferentially lost human chromosomes but had retained significant amounts of both mouse and human ribosomal genes (genes coding for the 18S, 5.8S, and 28S RNAs of ribosomes). However, the human ribosomal genes were repressed, and only mouse ribosomal genes were expressed. Soluble transcription extracts from the hybrids were able to initiate RNA synthesis accurately on a cloned mouse ribosomal gene but were unable to initiate accurately on a human ribosomal gene. This suggests that nucleolar dominance in these hybrids is due to the loss or inactivation of the gene for a specificity factor required to recognize the human ribosomal gene promoter.**

In hybrids between closely related species of both plants and animals, it is often observed that the ribosomal genes (coding for the 18S, 5.8S, and 28S RNA of mature ribosomes) of one species are transcriptionally dominant over the ribosomal genes of the other species (1–4). The ribosomal genes of the dominated species are not lost in the hybrids and under appropriate circumstances can be reactivated. In addition, dominance between closely related species does not show a maternal effect. The same species is dominant regardless of which furnishes the sperm and which furnishes the egg. Recent work on the developmental regulation of ribosomal gene expression in *Xenopus laevis* suggests that, for crosses involving that species, nucleolar dominance may be due to competition between the nontranscribed spacers for factors needed to activate the ribosomal gene promoter (5, 6).

A type of nucleolar dominance has also been described in somatic cell hybrids between distantly related species such as mouse and human (7, 8) and has been shown to also operate at the transcriptional level (9). These hybrids are characterized by the fact that many of the chromosomes of one partner are lost in the hybrid cells. Although at least some ribosomal genes from both species are often retained, the species whose chromosomes are lost in part is always the species whose residual ribosomal genes are transcriptionally repressed. Experiments described in this report show that nucleolar dominance in mouse–human hybrids can be due to the loss or inactivation of a gene for a transcriptional specificity factor. Such a mechanism is quite different from the mechanism that appears to be operating in closely related species hybrids such as between *Xenopus* species.

MATERIALS AND METHODS

Growth of Cells. NS-1 mouse myeloma cells and both hybrid cell lines (F-6 and A-9) were grown in RPMI medium (GIBCO) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 15% fetal calf serum (Armour, Chicago). HeLa cells were grown in minimal essential medium (GIBCO) supplemented with 5% newborn calf serum (M. A. Bioproducts, Walkersville, MD).

Nucleic Acid Extraction and Analysis. DNA was extracted from cells, digested with restriction enzymes, electrophoresed on agarose gels, blotted to nitrocellulose, and hybridized with nick-translated probes by using minor variations of standard procedures as described by Busby and Reeder (10).

For RNA analysis, total nucleic acid was glyoxalated (11), either with or without prior digestion with 20 μg of heat-treated RNase A per ml for 30 min at 37°C. All other details of electrophoresis, blotting, and hybridization with nick-translated probes were as described by Thomas (12).

Cloned Ribosomal DNA. pSalI-B was a gift from I. Grummt. It contains a 3.3-kilobase (kb) *Sal* I fragment of mouse ribosomal DNA (13) that includes the promoter, initiation site, and part of the external transcribed spacer. pHrC was subcloned from a larger fragment of human ribosomal DNA given to us by M. Crippa. pHrC is a 1.2-kb *Eco*RI–*Sal* I fragment that contains the promoter and part of the external transcribed spacer (14–16). To make hybridization probes, either pSalI-B or pHrC was nick-translated as described by Maniatis *et al.* (17). To make run-off templates for transcription assays, pSalI-B was digested with *Xho* I, whereas pHrC was digested with *Sal* I.

***In Vitro* Transcription Assays.** S-100 extracts were prepared as described by Weil *et al.* (18) with a slight modification. To the homogenate in hypotonic buffer was added 1/4 vol of 100 mM Hepes, pH 7.9/80% glycerol/500 mM KCl/17.5 mM $MgCl_2$/5 mM dithiothreitol/0.1 mM EDTA. The 100,000 × *g* supernatant was used without dialysis.

The reaction mixture (50 μl) contained 22 mM Hepes (pH 7.9), 12.5% glycerol, 5 mM $MgCl_2$, 80 mM KCl, 0.75 mM dithiothreitol, 600 μM (each) ATP, CTP, and UTP, 10 μCi of [α-^{32}P]GTP (ICN; 25 Ci/mmol; 1 Ci = 37 GBq), 200 μg of α-amanitin per ml, 1 μg of restriction enzyme-digested ribosomal gene plasmid, and various amounts of S-100 extract. After incubation for 60 min at 25°C, to the reaction mixture was added 350 μl of 0.3 M sodium acetate (pH 7), 1 mM EDTA, 0.3% Sarkosyl, and 100 μg of *Escherichia coli* tRNA per ml. The mixture was extracted with phenol and then with phenol/chloroform/isoamyl alcohol, 25:25:1 (vol/vol), followed by ethanol precipitation. The ethanol precipitate was dissolved by addition of 0.2 ml each of 0.2% $NaDodSO_4$ and 2 M ammonium acetate and was precipitated with ethanol (19). The resulting ethanol precipitate was dissolved in 0.2 ml of 0.3 M sodium acetate, pH 5.3/0.2% $NaDodSO_4$, to which 6.6 ml of ethanol was added. Approximately 12,000 cpm of acid-insoluble radioactivity was subjected to glyoxalation and agarose gel electrophoresis as described (12). The agarose gel was dried and exposed to x-ray film at −70°C with a Cronex Hi-plus screen.

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Abbreviations: kb, kilobase(s); bp, base pair(s).

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Derivation of Mouse–Human Hybrids. A line of mouse myeloma cells, NS-1, was fused with cells from human lymph nodes and a clonal hybrid line, F6, was selected for study. F6 contains 125 chromosomes, of which <10 are human. The F6 hybrid cell line was, in turn, fused with human spleen cells and a second clonal line, A9, was selected for study. Hybrid line A9 has about 130 chromosomes and about 10 of them are of human origin.

RESULTS

Hybrid Lines F6 and A9 Contain Both Mouse and Human Ribosomal Genes. Equal amounts of DNA from the parental mouse myeloma (NS-1), from both hybrid cell lines (F-6 and A-9), and from human (HeLa) cells were doubly digested with *Eco*RI and *Sal* I, blotted onto nitrocellulose, and hybridized with a radioactive probe specific for mouse ribosomal genes. As shown in Fig. 1*A*, the hybrid cells contain a concentration of mouse ribosomal genes close to that of the parental myeloma line. As expected, the radioactive probe did not crossreact with anything in the human (HeLa) genome.

In the reverse experiment, equal amounts of DNA from all four cell lines were digested with *Eco*RI and *Bam*HI, blotted, and probed with a human-specific probe (Fig. 1*B*). Both hybrid lines contain human ribosomal genes. Hybrid line A9 has 0.5–0.75 of the amount of ribosomal genes present in a HeLa cell, whereas line F6 has slightly less than half of the amount in a HeLa cell. The presence of two bands is probably due to a restriction site difference, which we have not investigated further.

Hybrid Lines F6 and A9 Transcribe Only Mouse Ribosomal RNA. Equal amounts of RNA from NS-1, F6, A9, and HeLa cell lines were electrophoresed on glyoxal gels, blotted, and probed with a mouse-specific probe that should only hybridize with the 5′ end of the external transcribed spacer of the precursor ribosomal RNA. As shown in Fig. 2*A*, this probe reacted with several bands in the NS-1, F6, and A9 lines but did not react with HeLa RNA. Prior treatment with RNase completely abolished the hybridization. The largest of the bands is close to the predicted size for the mouse ribosomal RNA precursor. Presumably the smaller bands are processing or breakdown products of the precursor. None of the bands coincides with mature 18S or 28S ribosomal RNA. In the reciprocal experiment (Fig. 2*B*), equal amounts of RNA from all four cell lines were blotted and probed with a human-specific probe. No human ribosomal RNA was detected in the hybrid cells.

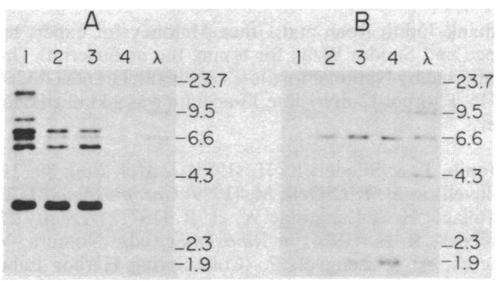

FIG. 1. Detection of both mouse and human ribosomal DNA in hybrid cells. (*A*) Hybridization with a mouse ribosomal DNA probe. DNA from each cell line (3 μg) was digested with *Eco*RI and *Sal* I, electrophoresed, blotted onto nitrocellulose, and hybridized with the mouse-specific ribosomal DNA present in pSalI-B. Lane 1, mouse (NS-1) DNA; lane 2, hybrid (F-6) DNA; lane 3, hybrid (A-9) DNA; lane 4, human (HeLa) DNA. (*B*) Hybridization with a human ribosomal DNA probe. DNA from each cell line (3 μg) was digested with *Eco*RI and *Bam*HI, electrophoresed, blotted onto nitrocellulose, and hybridized with the human-specific ribosomal DNA present in pHrC. Lane 1, mouse (NS-1) DNA; lane 2, hybrid (F-G) DNA; lane 3, hybrid (A-9) DNA; lane 4, human (HeLa) DNA. Phage DNA digested with *Hin*dIII was used as size markers (shown in kb) on both blots.

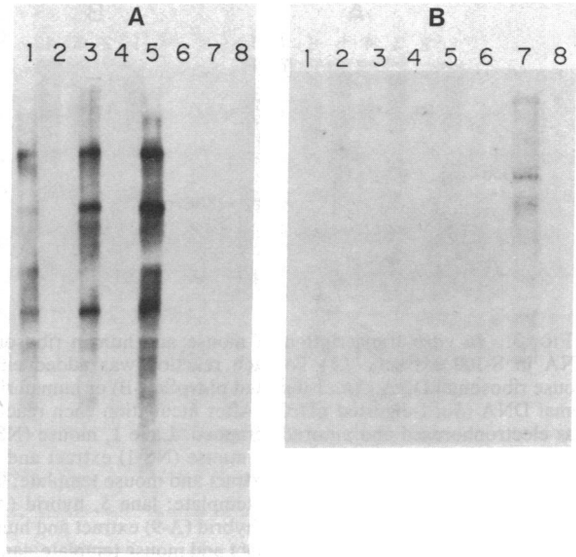

FIG. 2. Human precursor ribosomal RNA is absent in the hybrid cell lines. (*A*) Hybridization with a mouse ribosomal DNA probe. Total nucleic acid (10 μg) from each cell line was glyoxylated, electrophoresed, blotted onto nitrocellulose, and hybridized with the mouse-specific ribosomal DNA present in pSalI-B. Lane 1, mouse (NS-1) nucleic acid; lane 2, same as in lane 1 but pretreated with RNase before electrophoresis; lane 3, hybrid (F-6) nucleic acid; lane 4, same as in lane 3 with RNase; lane 5, hybrid (A-9) nucleic acid; lane 6, same as in lane 5 with RNase; lane 7, human (HeLa) nucleic acid; lane 8, same as in lane 7 with RNase. (*B*) Hybridization with a human ribosomal DNA probe. Same as *A* except a parallel blot was hybridized with the human-specific ribosomal DNA present in pHrC.

We conclude that the mouse–human hybrid lines F6 and A9 have characteristics similar to others that have been reported in the literature. Mouse chromosomes have been preferentially retained and, although both species of ribosomal genes are present, only the mouse ribosomal genes are expressed. Because our probes are specific for the primary transcripts of these genes the dominance is likely to be at the level of transcription rather than differential processing.

S-100 Extracts of the Hybrid Cell Lines Lack a Human Transcriptional Specificity Factor(s). The transcriptional machinery for ribosomal genes has evolved with sufficient rapidity that genes from the human are not transcribed by extracts from the mouse in the commonly used S-100 run-off assay (20, 21). This specificity is not absolute because, under certain circumstances, such widely separated species as mouse and *Xenopus* can be made to crossreact (22). However, as it is usually employed, the S-100 run-off assay appears to be a valid method to assay for species-specific transcriptional factors. This conclusion is strengthened by the fact, as Mishima *et al.* (21) have shown, that human species specificity can be conferred on a mouse extract by supplementing the mouse extract with a single human fraction that binds tightly to phosphocellulose.

With this background in mind, we made S-100 extracts from all four cell lines and tested their ability to transcribe mouse and human ribosomal genes in the run-off assay. The results are shown in Fig. 3*A* and *B*. In Fig. 3*A*, lanes 1, 3, 5, and 7, a truncated mouse ribosomal gene template was added to extracts from NS-1, F6, A9, and HeLa cells, respectively. The parental NS-1 cells as well as both hybrid cell lines (F6 and A9) actively transcribed mouse ribosomal DNA to yield the expected run-off product of 1,300 base pairs (bp) (23). In contrast, the HeLa extract gave no dis-

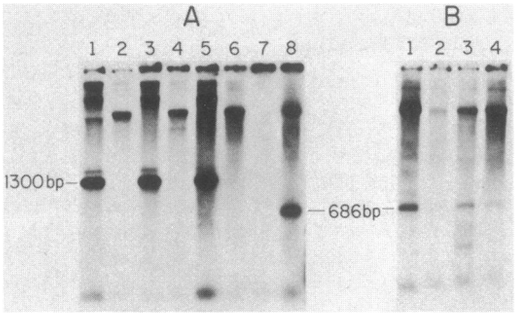

FIG. 3. *In vitro* transcription of mouse and human ribosomal DNA in S-100 extracts. (*A*) To each reaction was added either mouse ribosomal DNA (*Xho* I-digested pMrpSalI-B) or human ribosomal DNA (*Sal* I-digested pHrC). After incubation each reaction was electrophoresed and autoradiographed. Lane 1, mouse (NS-1) extract and mouse template; lane 2, mouse (NS-1) extract and human template; lane 3, hybrid (F-6) extract and mouse template; lane 4, hybrid (F-6) extract and human template; lane 5, hybrid (A-9) extract and mouse template; lane 6, hybrid (A-9) extract and human template; lane 7, human (HeLa) extract and mouse template; lane 8, human (HeLa) extract and human template. Specific initiation on the mouse template results in a run-off product 1,300 bp long (22). Specific initiation on the human template yields a product 686 bp long (14–16). (*B*) Lane 1, human (HeLa) extract and human template; lane 2, human/mouse extracts (1:1) and human template; lane 3, human/F-6 extract (1:1) and human template; lane 4, human/A-9 extract (1:1) and human template.

crete band at all with mouse ribosomal DNA. In Fig. 3*A*, lanes 2, 4, 6, and 8, extracts from the same four cell lines were challenged with a truncated human ribosomal gene template. Only the HeLa extract was able to specifically initiate on the human template and produce the expected run-off product of 686 bp (14–16).

In Fig. 3*B*, we mixed equal amounts of HeLa extract with the NS-1, F6, and A9 extracts and challenged them with the human ribosomal gene. The specific human transcription product was clearly visible in each mixture. This would seem to rule out the presence of an inhibitor of human transcription in the hybrid extracts or of a nuclease that specifically destroys human ribosomal gene transcripts.

We conclude that the simplest and most likely explanation for these results is that the hybrid cell lines lack at least one transcriptional factor that would allow recognition of the human ribosomal gene promoter. At present, we cannot tell if this lack is due to loss of a gene or due to inactivation of the gene or its product.

DISCUSSION

In previous discussions of nucleolar dominance, closely related hybrids (such as between *Xenopus* species) and distantly related hybrids (such as mouse–human somatic hybrids) have been treated together because superficially they appeared to be similar. The results reported here and elsewhere (5, 6) suggest that, in fact, the two types of nucleolar dominance operate by quite different mechanisms.

In the case of closely related species, such as *X. laevis* and *Xenopus borealis*, ribosomal genes from either species are transcribed with similar efficiency when microinjected into *X. laevis* oocytes (24). This suggests that the polymerase I transcription machinery has not evolved very far apart between these two species and that transcription factors from *X. laevis* will function on either species of ribosomal gene. Nonetheless, when the two species are mated, only *X. laevis* ribosomal genes turn on in the early embryo (1, 25). We have presented data elsewhere (5, 6) arguing that this dominance is due to competition by a repetitive element in the *Xenopus* spacer for binding of a transcription factor needed to activate the promoter of either species. The *X. laevis* spacer has more of these repetitive elements, outcompetes the *X. borealis* spacer for binding, and thus *X. laevis* ribosomal genes are transcriptionally dominant over *X. borealis* ribosomal genes under conditions in which the factor is limiting.

A different situation exists for distantly related species such as mouse and human. In this case, the polymerase I transcriptional machinery has evolved sufficiently far apart that the ribosomal genes of one species are not transcribed in extracts from the other species (20, 21). The work of Mishima *et al.* (21) suggests that this specificity may reside in a single transcription factor that has diverged between mouse and human. The results presented here suggest that nucleolar dominance in mouse–human hybrids is simply due to the loss or inactivation of the gene for the human specificity factor. Similar results have been obtained by R. Meisfeld (personal communication) for three other independently derived mouse–human hybrid lines.

Mouse–human somatic hybrids in some cases will segregate mouse chromosomes and in other cases will segregate human chromosomes, depending on just which cell types were used in the initial fusion (discussed in ref. 8). In every case described so far, the species whose chromosomes are retained is the species whose ribosomal genes are dominant in transcription. This supports the notion that failure of one set of ribosomal genes to transcribe is simply due to the loss of a gene for a transcription factor. However, this simple explanation does not agree with reports that in some hybrid lines the inactive ribosomal genes can be reactivated either by infecting the cells with simian virus 40 (26, 27) or by treating them with the tumor promoter phorbol 12-myristate 13-acetate (28). It is possible that the specificity gene is inactivated in some cell lines and lost in others, depending on just which chromosomes are discarded. In this case, only certain hybrid lines would have the potential for reactivation of the inactive ribosomal DNA.

We have tried reactivating the human ribosomal genes in our hybrid lines by treating them with a variety of phorbol 12-myristate 13-acetate concentrations and so far we have failed to observe any reactivation (data not shown). It would be of interest to examine the transcriptional specificity of extracts from a hybrid cell line that is capable of reactivation.

Regardless of the final resolution of the reactivation question, nucleolar dominance between closely related species appears to be due to a quite different mechanism than dominance between distantly related species.

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