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# Transcription Stimulates Homologous Recombination in Mammalian Cells

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**Transcription stimulates homologous recombination in *Saccharomyces cerevisiae* and has been implicated in the control of recombinational events during the development of mammalian immune systems. Here, we describe a plasmid-based system in which an inducible promoter from the mouse mammary tumor virus is located upstream of heteroallelic neomycin genes carried on two plasmids. Pairs of plasmids are introduced into Chinese hamster ovary cells by electroporation, and recombination is monitored by scoring colonies resistant to the aminoglycoside G418. When transcription is induced with dexamethasone, a synthetic glucocorticoid hormone, and double-strand breaks are introduced at mutation sites, recombination is stimulated sixfold over noninduced levels. Inducing transcription in circular substrates or in substrates cleaved at sites distant from the mutations has no detectable effect on recombination between neomycin genes. Results are presented that are consistent with the observed stimulation of recombination occurring before plasmids integrate into the cellular DNA. Our results are discussed in relation to molecular models for extrachromosomal recombination in mammalian cells.**

Genetic recombination in eucaryotic cells is induced by DNA-damaging agents, such as UV light, X rays, and chemicals (e.g., methylmethane sulfonate) (reviewed in reference 37). Recombination is also stimulated by enzymatically produced double-strand breaks (DSBs) (5–7, 15, 19, 21, 23, 25–28, 32, 33, 35, 46, 49; reviewed in reference 42). Models to explain how DNA damage might stimulate recombination include those that propose interactions between DNA ends with homologous DNA elsewhere in the genome (33, 34, 36, 44; reviewed in references 42 and 44) and those that propose exonucleolytic degradation, exposing complementary single-stranded regions (7, 25–28, 49). The ends may be created directly by the damaging agent (e.g., primary radiation damage) or by enzymatic processes during DNA repair (e.g., single-strand gap production during excision repair of thymine dimers). Induced recombination has also been studied extensively in procaryotes (see reference 9 for a review).

Recombination in *Saccharomyces cerevisiae* is also stimulated by transcription. Transcription by RNA polymerase I stimulates recombination between repeated genes in yeast cells (18, 48); inter- and intrachromosomal mitotic recombination increases 7- to 10-fold when the yeast ribosomal RNA promoter is located upstream of repeated elements, but only when both duplicated regions are transcribed. In addition, transcription mediated by the inducible *GAL1-10* promoter stimulates recombination between repeated DNA in yeast cells, showing that this effect is not specific for RNA polymerase I-mediated transcription (45; J. Nickoloff, unpublished observations).

Recently, it has been suggested that transcription levels may influence homologous recombination in mammalian cells (16, 30). In an early study, no difference in homologous recombination frequencies was observed when exogenous DNA was targeted to transcriptionally active or silent β-globin loci (40). Indirect evidence suggesting that transcription enhances recombination, however, has come from studies on the development of the mammalian immune system. During the development of immunoglobulin genes in B cells and T-cell receptor genes, specific recombination events mediate the assembly of mature genes from component gene segments (reviewed in references 1 and 47). Alt et al. (2) proposed that some of these events depend on the introduction of specific DSBs near the recombining elements. Alt et al. (1) and Blackwell et al. (4) showed that transcriptionally active immunoglobulin gene segments recombine at high frequencies and have proposed that this process may be regulated at the level of transcription, i.e., that the accessibility of a chromosomal region may control whether the recombinase cleaves the target DNA and that accessibility may be controlled by transcription. An alternative model is that transcription may be controlled by factors that also influence target accessibility. Similar proposals have been made with respect to the assembly of T-cell receptor genes (43).

In this report, we describe a system in which transcription of heteroallelic neomycin (*neo*) genes on shuttle vectors stimulates homologous recombination in Chinese hamster ovary (CHO) cells. Transcription from the mouse mammary tumor virus (MMTV) promoter is induced by the glucocorticoid dexamethasone (dex) (38). We demonstrate that the induction of transcription stimulates recombination between substrates having DSBs at mutation sites. These results are discussed in relation to current models of recombination mechanisms.

## MATERIALS AND METHODS

**Plasmid DNA constructions and preparations.** Plasmids were constructed by standard procedures (29) and are shown in Fig. 1. Plasmid DNAs were prepared by the alkaline lysis method of Maniatis et al. (29). Derivatives of plasmid pMSG (Pharmacia) were constructed as follows. *Sal*I linkers were added to an 1,100-base-pair (bp) *Hin*dIII-*Sma*I fragment of pSV2neo (41) carrying the *neo* coding region. This fragment was inserted into pUC19, and the pUC19 *Eco*RI site was

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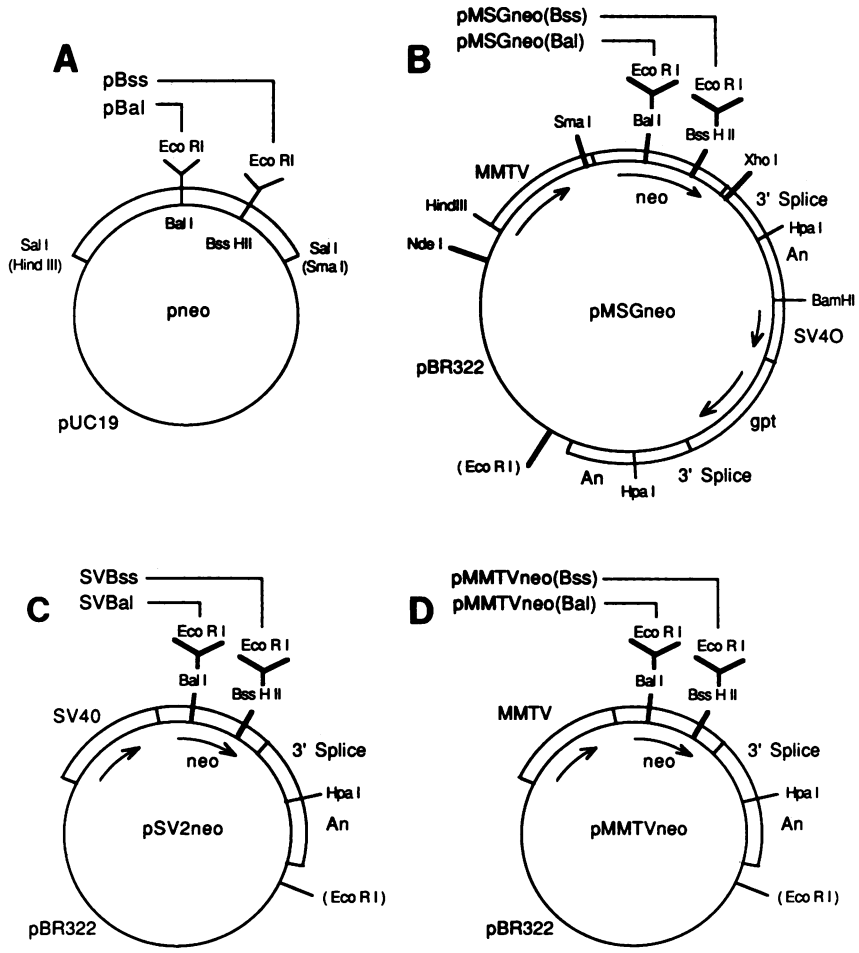


FIG. 1. Structures of plasmids used in this study. All plasmids carry an 1,100-base-pair *Hin*dIII-*Sma*I fragment with *neo* coding region from pSV2neo. (A) pneo carries the *Hin*dIII-*Sma*I *neo* fragment from pSV2neo inserted into the *Sal*I site of pUC19 (shown by the open box). The polylinker *Eco*RI site is deleted in pneo, and both *Hin*dIII and *Sma*I sites (shown in parentheses) have been destroyed. Two derivatives, pBal and pBss, were constructed by inserting *Eco*RI linkers into the *Bal*I and *Bss*HII sites of pneo, respectively. (B) Structures of pMSGneo derivatives. pMSGneo carries the *Sal*I *neo* fragment downstream of the MMTV promoter and the selectable SV40 *gpt* gene on a derivative of pBR322 (shown by the single line). Mutant *neo* genes from pBal and pBss were inserted into pMSG to create pMSGneo(Bal) and pMSGneo(Bss). The *Eco*RI site in pBR322 DNA (shown in parentheses) is missing in pMSGneo(Bal)-R and pMSGneo(Bss)-R. (C) Structures of pSV2neo derivatives. SVBal and SVBss were constructed from a derivative of pSV2neo missing the *Eco*RI site (in parentheses) by inserting *Eco*RI linkers into the *Bal*I and *Bss*HII sites, respectively. (D) Structures of pMMTVneo derivatives. These plasmids were constructed from corresponding pMSGneo plasmids by deleting the 2.25-kbp *Hpa*I fragment. Bold lines indicate the locations of restriction sites cleaved in recombination experiments.

destroyed, creating pneo. Ten-base-pair *Eco*RI linkers were inserted into the unique *Bal*I and *Bss*HII sites in the *neo* coding region in pneo, creating plasmids pBal and pBss, respectively. The mutant *neo* genes in these plasmids were inserted into the *Sal*I site of pMSG, producing plasmids pMSGneo(Bal) and pMSGneo(Bss). These plasmids contain two *Eco*RI sites: one in the *neo* gene and one in pBR322 DNA of pMSG. Two related plasmids with unique *Eco*RI sites in the *neo* gene were produced by inserting the mutant *neo* genes into a derivative of pMSG lacking the *Eco*RI site. These plasmids are called pMSGneo(Bal)-R and pMSGneo (Bss)-R. Plasmid pMSGneo, with a wild-type *neo* gene (*neo*$^+$), was produced by inserting the *neo* fragment from pneo into the *Sal*I site of pMSG. Plasmids pMMTVneo, pMMTVneo(Bal), and pMMTVneo(Bss) were constructed by deleting the 2.25-kbp *Hpa*I fragment from the appropriate pMSGneo derivatives; the *Hpa*I fragment contains the simian virus 40 (SV40) promoter and the *Escherichia coli gpt* gene. Plasmids SVBal and SVBss are derivatives of pSV2neo (41), with *Eco*RI linker mutations in the *Bal*I and *Bss*HII sites, respectively, and lack the pBR322 *Eco*RI site.

**Genomic DNA preparation and Southern hybridization analysis.** Genomic DNAs were prepared as follows: confluent monolayers in 100-mm (diameter) dishes were lysed in 2.0 ml of 20 mM Tris (pH 7.6)–100 mM NaCl–10 mM EDTA (pH 8.0)–0.5% sodium dodecyl sulfate–0.1 mg of proteinase K per ml for 2 h at 37°C. Lysates were extracted twice with phenol-$CHCl_3$-isoamyl alcohol (25:24:1) and twice with $CHCl_3$-isoamyl alcohol (24:1). DNAs were precipitated with 2 ml of ethanol, suspended in 0.5 ml of 10 mM Tris (pH 8.0)–1 mM EDTA (TE) to which 0.05 ml of RNase A (10 mg/ml) was added, and incubated at 37°C for 1 h. DNAs were precipitated with ethanol and suspended in 0.25 ml of Tris-EDTA. Hybridization analysis was performed by using 10-μg portions of each DNA, which were electrophoresed on 0.8% agarose gels and transferred to a Zetabind membrane

by using the recommendations of the manufacturer. Bound DNAs were hybridized to a 1.1-kbp *SalI* fragment containing the *neo* gene and $^{32}P$ labeled by random primer extension (Boehringer Mannheim).

**RNA preparation and Northern (RNA blotting) hybridization analysis.** Total RNA was prepared from confluent monolayers of a G418$^r$ transfectant as follows. Cells were harvested by treatment with trypsin and washed twice with phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$ [pH 7.2]). Cells were suspended in 0.25 ml of 10 mM Tris (pH 7.8)–150 mM NaCl–10 mM vanadyl ribonucleoside complex, incubated on ice for 1 min, and lysed with 0.028 ml of 10% Nonidet P-40. Cell debris was removed by centrifugation for 2 min at 16,000 × *g*. Supernatants were added to 0.25 ml of 40 mM Tris (pH 7.8)–40 mM EDTA–700 mM NaCl–2% sodium dodecyl sulfate, vortexed, and then extracted twice with phenol-$CHCl_3$-isoamyl alcohol (25:24:1) and twice with $CHCl_3$-isoamyl alcohol (24:1). RNAs were precipitated with 0.5 ml of ethanol and suspended in 0.05 ml of $H_2O$. RNAs were electrophoresed on 1.0% agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized to $^{32}P$-labeled *neo* or *gpt*, as described by Maniatis et al. (29). The *gpt* fragment consisted of a gel-purified 1.05-kbp *Hin*dIII-*Apa*I fragment from pSV2gpt (31).

**Cell culture and electroporation.** Chinese hamster ovary cells (strain K1c) were maintained in α minimum essential medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. DNA was introduced by electroporation as described by Chu et al. (8), with the following modifications. Following restriction endonuclease digestion, enzymes were heat inactivated in a solution containing 0.05% sodium dodecyl sulfate, and the DNA was purified by passage through a Sepharose CL-6B spin column (Pharmacia). Prior to electroporation, cells were harvested and suspended in growth medium and cell titers were determined with a Coulter counter. Cells were then washed once in phosphate-buffered saline and suspended in phosphate-buffered saline at a concentration of $5.33 \times 10^6$ cells per ml. Portions (5 μg) of each DNA plus $4 \times 10^6$ cells in a volume of 0.8 ml were transferred to a Bio-Rad electroporation cuvette and shocked with 300 V at 960 μF with a Bio-Rad Gene Pulser. Cells, maintained at room temperature during harvesting and electroporation, were transferred to 25 ml of prewarmed growth medium. The colony-forming ability of treated cells was 30 to 50% of that of untreated cells.

After each electroporation, two 2.5-ml samples were plated for later selection with medium containing MAX (10 μg of mycophenolic acid per ml, 12.5 μg of adenine per ml, 250 μg of xanthine per ml), and two 10-ml samples were plated for later selection with G418 (500 μg/ml, 50% active [GIBCO]) medium. Dex (final concentration, 1.0 μM) was added to two of the four plates 1 to 2 h after electroporation. All media were replaced with selective media containing either MAX or G418 (but without dex) 24 h after electroporation. After 10 days in selective medium, colonies were stained with 1% crystal violet in 70% ethanol.

Recombination frequencies were calculated as the ratio of G418-resistant (G418$^r$) colonies to the total number of live cells plated in G418 medium. Transfection frequencies for the *gpt* gene carried by pMSG derivatives were calculated as the ratio of MAX-resistant (MAX$^r$) colonies to live cells plated. The live-cell count was determined by plating dilutions of shocked cells in nonselective medium.

## RESULTS

**Experimental design.** Plasmid pMSG (Pharmacia) is a derivative of pMDSG (24). pMSG contains the selectable *E. coli gpt* gene (31) flanked by the SV40 early promoter and SV40 3′ splicing and polyadenylation signals. Cells transfected with *gpt* can be selected by growth in MAX medium. pMSG also carries an expression cassette, consisting of a polylinker flanked by the inducible MMTV promoter (38), and 3′ splice and polyadenylation signals from SV40. Derivatives of pMSG were constructed with *neo*$^+$ or mutant *neo* genes under MMTV promoter control (see Materials and Methods and Fig. 1).

To examine recombination, pairs of plasmids carrying heteroallelic mutant *neo* genes were mixed and electroporated into mammalian cells. Recombination between plasmids can create a *neo*$^+$ gene. Subsequent integration of a recombinant plasmid into the cellular DNA leads to a stable G418$^r$ transfectant. Because of the presence of a second selectable marker (*gpt*) on pMSGneo derivatives, both recombinant (G418$^r$) and nonrecombinant (G418-sensitive) plasmids confer MAX$^r$ to stably transfected cells. The frequency of MAX$^r$ colonies would therefore be expected to be useful as an internal control for transfection efficiency under the same conditions employed to examine recombination. However, unexpected complications were encountered when markers driven by the SV40 early promoter were used for this purpose (see below).

A series of control experiments were performed to characterize the effects of dex on MMTV *neo* and SV40 *gpt* transcription levels, the requirements for added dex in selecting G418$^r$ MMTV *neo* transfectants, and the reversion frequencies of the mutant MMTV *neo* genes used in recombination assays.

**Effects of dexamethasone on transcription levels of MMTV *neo* and SV40 *gpt*.** Transcription levels of integrated copies of the MMTV *neo* and SV40 *gpt* genes were monitored in the presence and absence of dex by Northern analysis. RNA was isolated from a G418$^r$ transfectant carrying one or two copies of MMTV *neo* grown with and without dex. Dex strongly enhanced the level of MMTV *neo* transcription (Fig. 2). In contrast, dex has an inhibitory effect on transcription of the SV40 *gpt* gene. Dex-mediated reduction of SV40 transcription is discussed further below.

Attempts to measure transcription levels of MMTV *neo* and SV40 *gpt* prior to integration (i.e., transient expression levels) were made in newly transfected cells, but without success (data not shown). This might have been due to two compounding factors inherent in electroporation-mediated gene transfer: (i) small numbers of cells receiving DNA and (ii) small amounts of DNA taken up by each cell (see Discussion).

**Expression of G418$^r$ by pMSGneo-transformed cells in the absence of dexamethasone.** Control experiments were performed with pMSGneo to determine if dex is required to confer G418$^r$ to stably transfected cells. Plasmid pMSGneo, carrying wild-type *neo* and *gpt* genes, was digested with *Nde*I, which cleaves once in pBR322 DNA, and transfected into CHO cells. Five independent MAX$^r$ transfectants were recovered, and inoculated into G418 medium with or without dex. Each cell line was G418$^r$ in the presence and absence of dex. Thus, the MMTV promoter is sufficiently active in the absence of dex to confer resistance to G418 in cells transfected with pMSGneo. Although the copy number of MMTV *neo* genes in these lines was not determined, evidence

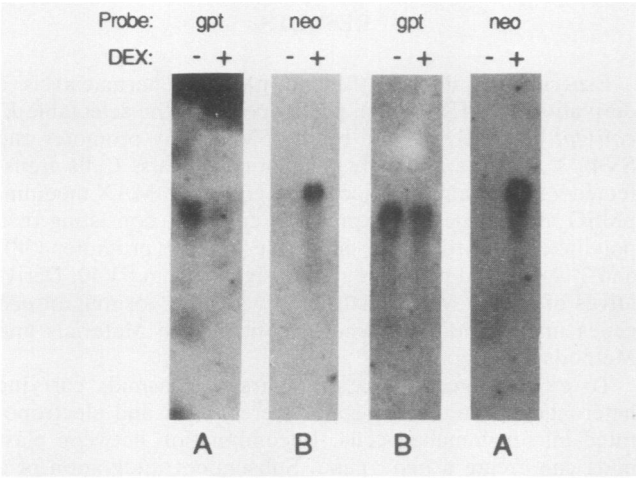


FIG. 2. Expression of *neo* and *gpt* in a G418$^r$ line produced by recombination between pMSGneo(Bal)-R and pMSGneo(Bss)-R cleaved with *Eco*RI. Southern analysis of this line indicates that it carries 1 to 2 copies of MMTV *neo* (Fig. 4). This line was grown into mass culture, and cells were plated on two 100-mm (diameter) dishes. Two days prior to confluency, 1 μM dex was added to one of the plates, and both plates were incubated for 2 days. RNA was isolated and Northern hybridization analysis was performed as described in Materials and Methods. Two replicate blots (A and B) with RNA from dex-treated and untreated cells were prepared. Blot A was hybridized to [$^{32}$P]*gpt* and blot B was hybridized to [$^{32}$P]*neo* (left two panels). Radioactive probes were stripped from both blots with boiling $H_2O$ for 5 min, and the blots were rehybridized, but with probes switched (right two panels). Faint *neo* signals can be detected in the lanes without dex after longer exposure (not shown). −, Dex absent; +, dex present.

presented below indicates that single copies of MMTV *neo* are sufficient to confer G418$^r$ without added dex.

**Mutation reversion in pMSGneo derivatives.** The four derivatives of pMSGneo with mutant *neo* genes were introduced into cells individually to determine reversion frequencies for each mutation. Electroporation, addition of dex, and selection with MAX- or G418-containing media were performed as described in Materials and Methods. Experiments were performed three times with each plasmid. No G418$^r$ colonies arose when plasmids were cleaved at sites other than the *Eco*RI mutation sites or when the plasmids were undigested. Only one G418$^r$ colony arose with *Eco*RI-digested pMSGneo(Bss)-R in three repetitions, giving a reversion frequency of $10^{-6}$; no revertants were obtained with other *Eco*RI-digested plasmids (reversion frequencies less than $10^{-6}$).

**Dexamethasone-stimulated recombination between heteroallelic genes.** The following experiments were performed to determine the effects of transcriptional activity through mutant *neo* genes on recombination between these genes. Circular or linear heteroallelic derivatives of pMSGneo were mixed and introduced into CHO cells. Dex was added to half of the cultures before MAX or G418 selections were applied, as described in Materials and Methods. For each cross, both plasmids were treated with the same enzyme (see Fig. 1 for locations of cleavage sites). The results of these experiments are presented in Table 1.

Similar results were obtained for both pMSGneo(Bal) × pMSGneo(Bss) crosses and pMSGneo(Bal)-R × pMSGneo (Bss)-R crosses. Cleaving plasmids increased the transfection frequency of SV40 *gpt*, as shown by the increase in MAX$^r$ colonies relative to levels seen with circular plasmids. Dex had no effect on recombination frequencies when plasmids were cleaved in pBR322 DNA (*Nde*I) or at other locations outside the *neo* gene (*Xho*I or *Sma*I). As expected, DSBs at mutation sites (by digestion with *Eco*RI) increased recombination over levels observed with circular substrates (23). Adding dex enhanced recombination an additional sixfold. Although standard deviations are large in these experiments, increased recombination frequencies in dex-treated cultures were shown to be significant ($P[t] < 0.05$).

Increases in the frequency of G418$^r$ colonies with dex treatment might have resulted from factors other than transcriptional stimulation of recombination. For example, dex-induced transcription might permit cells containing single or few integrated *neo*$^+$ genes to survive G418 selection, while *neo* expression in these cells might be too low to confer resistance without added dex. In this view, the few G418$^r$

TABLE 1. Dex-induced recombination between pMSGneo derivatives

| Derivatives and enzyme[a] | n[b] | No. of colonies[c] | | | | Frequency, $10^5$[d] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MAX$^r$ | | G418$^r$ | | MAX$^r$ | | G418$^r$ | |
| | | Dex absent | Dex present | Dex absent | Dex present | Dex absent | Dex present | Dex absent | Dex present |
| pMSGneo(Bal) and pMSGneo(Bss) | | | | | | | | | |
| None | 4 | 58 ± 12 | 27 ± 18 | 1.0 ± 0.8 | 0.5 ± 1.0 | 186 ± 93 | 91 ± 67 | 1.8 ± 1.3 | 0.6 ± 1.1 |
| *Eco*RI | 6 | 240 ± 48 | 109 ± 33 | 5.8 ± 4.0 | 37 ± 19 | 341 ± 145 | 165 ± 87 | 3.0 ± 2.0 | 19 ± 9.2 |
| *Nde*I | 2 | 429 | 176 | 0.5 | 0.5 | 315 | 130 | 0.2 | 0.2 |
| pMSGneo(Bal)-R and pMSGneo(Bss)-R | | | | | | | | | |
| *Eco*RI | 6 | 197 ± 28 | 98 ± 18 | 8.4 ± 4.8 | 25 ± 8.2 | 515 ± 267 | 245 ± 98 | 6.4 ± 6.2 | 36 ± 28 |
| *Xho*I | 2 | 207 | 135 | 3.0 | 0 | 571 | 381 | 2.0 | 0 |
| *Sma*I | 2 | 140 | 44 | 0 | 0 | 521 | 171 | 0 | 0 |

[a] Enzymes used to cleave each of the two pMSGneo derivatives. *Eco*RI cleaves *neo* genes at mutation sites and in pBR322 DNA in pMSGneo(Bal) and pMSGneo(Bss) but only in *neo* genes in derivatives lacking the pBR322 *Eco*RI site.

[b] Number of times each experiment was performed.

[c] Average numbers ± standard deviations of MAX$^r$ and G418$^r$ colonies (with and without dex treatment) are given. In those cases for which only two determinations were made, only the average values are given.

[d] Average frequencies ± standard deviations of MAX$^r$ transfectants and G418$^r$ recombinants (with and without dex treatment) were calculated as described in Materials and Methods. Only average values are shown for experiments performed twice.

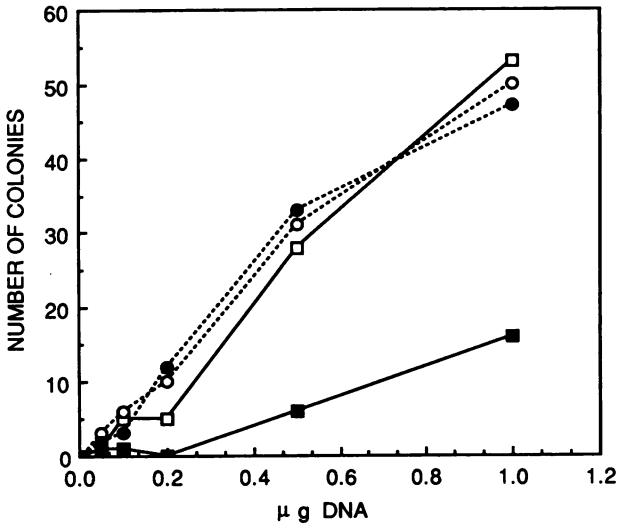


FIG. 3. Transfections with limiting dilutions of pMSGneo. Cells were transfected with 0.05, 0.1, 0.2, 0.5, or 1.0 μg of pMSGneo digested with *Nde*I. G418$^r$ and MAX$^r$ colonies were selected with and without prior dex treatment as described in Materials and Methods. □ and ■, MAX$^r$ values; ○ and ●, G418$^r$ values; ● and ■, dex-treated cells; ○ and □, untreated cells. Average values from two determinations are shown.

colonies that arise in the absence of dex are resistant to G418 because they contain greater numbers of *neo*$^+$ genes. Two approaches were taken to determine whether dex stimulates recombination or simply permits selection of transfectants with low *neo*$^+$ copy numbers.

In the first approach, cells were transfected with limiting dilutions of pMSGneo. We reasoned that as the concentration of pMSGneo was reduced, transfectants might carry fewer copies of pMSGneo. If dex treatment permits selection of low-copy-number transfectants, more G418$^r$ colonies would arise in the presence of dex than in its absence at pMSGneo concentrations that yield low-copy-number transfectants. (An alternative explanation is that lower transfection frequencies at low pMSGneo concentrations result from fewer cells receiving similar amounts of DNA at each DNA concentration. This possibility, however, circumvents the question of dex-dependent rescue of low-copy-number transfectants.)

pMSGneo was digested with *Nde*I, and dilutions were electroporated into CHO cells. Both MAX$^r$ and G418$^r$ colonies were selected with and without prior dex treatment as above. The results are shown in Fig. 3. The same number of MAX$^r$ and G418$^r$ transfectants arose at each DNA concentration in the absence of dex. The same number of G418$^r$ transfectants arose with or without dex treatment, suggesting that with DNA concentrations that give few or no transfectants, and possibly few *neo*$^+$ copies per transfectant, dex does not lead to increased recovery of G418$^r$ transfectants. Unexpectedly, the linked *gpt* and *neo* genes yielded different frequencies of MAX$^r$ and G418$^r$ transfectants in the presence of dex. Possible explanations for this result are given in the Discussion.

In the second approach, we analyzed the copy number of integrated *neo*$^+$ genes in G418$^r$ transfectants produced by homologous recombination between pMSGneo(Bal)-R and pMSGneo(Bss)-R in the presence and absence of dex. As shown in Fig. 4, G418$^r$ cell lines isolated from dex-treated cells have more *neo*$^+$ copies as well as more copies of nonrecombinant (mutant *neo*) genes. We conclude that dex treatment stimulates homologous recombination; dex treatment is not required for selection of transfectants carrying a single copy of a *neo*$^+$ gene.

Dex might increase recombination via nonspecific effects. To rule out this possibility, we monitored recombination between mutant derivatives of pSV2neo (SVBal and SVBss [Fig. 1]). As above, plasmids were cleaved at the unique *Eco*RI linker mutations before electroporation. The results for two experiments are shown in Table 2. No increases in

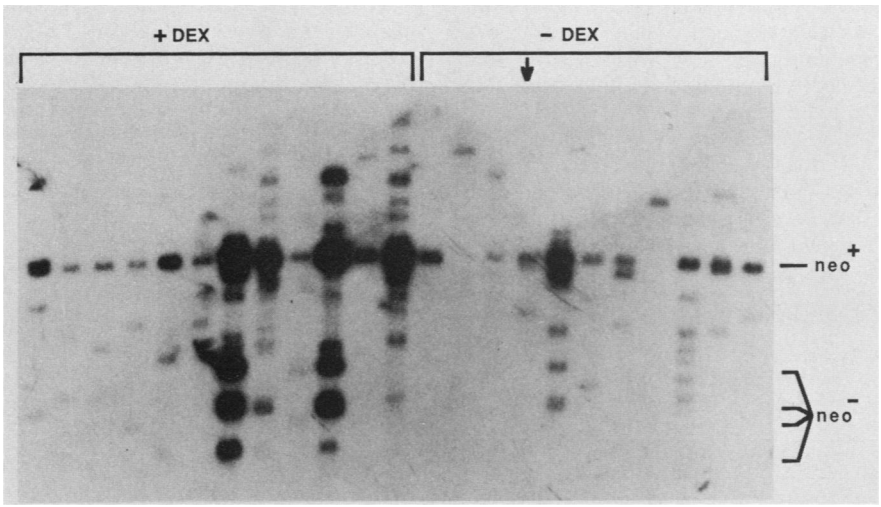


FIG. 4. Determination of the copy number of *neo*$^+$ genes in G418$^r$ transfectants with and without dex treatment. Genomic DNAs from 12 G418$^r$ isolates after dex treatment and 11 G418$^r$ isolates without dex treatment were digested with *Hin*dIII, *Bam*HI, and *Eco*RI and analyzed by Southern hybridization as described in Materials and Methods. Recombinant (*neo*$^+$) strains usually carry a 3.4-kbp *Hin*dIII-*Bam*HI fragment that is not cleaved by *Eco*RI (*neo*$^+$). Integrated nonrecombinant (*neo*$^-$) plasmids may also have the 3.4-kbp *Hin*dIII-*Bam*HI fragment, but this fragment is cleaved by *Eco*RI, producing four smaller fragments indicated by brackets (2.1, 1.8, 1.6, and 1.3 kbp). Alternative fragments are possible if plasmids integrate at sites between the *Hin*dIII and *Bam*HI sites. The cell line marked by the arrow was used in Northern analyses (Fig. 2).

TABLE 2. Effect of dex on recombination between SVBal and SVBss

| Expt no. | No. of G418$^r$ colonies[a] | | Frequency, $10^{5}$[b] | |
|---|---|---|---|---|
| | Dex absent | Dex present | Dex absent | Dex present |
| 1 | 156 | 41 | 33 | 8.8 |
| 2 | 149 | 51 | 42 | 14.5 |

[a] Number of G418$^r$ colonies formed in crosses between SVBal and SVBss in the presence or absence of dex. In both experiments, plasmids were cleaved at mutation sites with *Eco*RI prior to introduction into cells.

[b] Recombination frequencies were calculated as described in Materials and Methods.

recombination frequency were found with pSV2neo derivatives upon dex treatment; instead, the frequency of G418$^r$ colonies was reduced about threefold (this reduction is not due to reduced viability of dex-treated cells; see Discussion). Thus, dex stimulates recombination specifically between alleles driven by the MMTV promoter.

Although the increased recombination level between MMTV *neo* genes is MMTV promoter dependent, the results presented above do not rule out indirect effects due to promoter occlusion. Previous studies have shown that a strong promoter can suppress transcription of weak upstream or downstream promoters (11, 12, 17). In our system, dex may be indirectly stimulating transcription from the MMTV promoter by inhibiting the strong SV40 promoter. We examined MMTV promoter-specific dex effects in the absence of the SV40 promoter by using mutant derivatives of pMMTVneo (Fig. 1). The results (Table 3) indicate that dex-induced transcription from the MMTV promoter is sufficient to stimulate interplasmid recombination.

**Observed enhancement of recombination with dexamethasone treatment may occur before plasmids integrate into chromosomal DNA.** The following experiments were performed to determine if the observed stimulation of recombination occurs before or after plasmid integration. pMSGneo (Bal)-R and pMSGneo(Bss)-R were cleaved with *Eco*RI and electroporated into CHO cells. Eleven days later, 24 independent MAX$^r$ transfectants were recovered and grown in MAX medium for six days. Cells from each transfectant culture were treated as follows: approximately 200 cells were plated in G418 medium, with and without prior dex treatment, and in nonselective medium, without prior dex treatment. Additionally, approximately $1.5 \times 10^5$ cells, also with and without prior dex treatment, were plated in G418 medium. Seventeen of twenty-four transfectants were G418$^s$. Treatment with dex did not produce G418$^r$ colonies in these G418$^s$ cultures. The seven remaining MAX$^r$ transfectants were G418$^r$, with or without dex. These cultures gave similar numbers of colonies in both G418 and nonselective media (data not shown).

TABLE 3. Effect of dex on recombination between pMMTVBal and pMMTVBss

| Expt no. | No. of G418$^r$ colonies[a] | | Frequency, $10^{5}$[b] | |
|---|---|---|---|---|
| | Dex absent | Dex present | Dex absent | Dex present |
| 1 | 1 | 10 | 36 | 360 |
| 2 | 1 | 11 | 28 | 310 |
| 3 | 2 | 15 | 64 | 480 |

[a] Number of G418$^r$ colonies formed in crosses between pMMTVBal and pMMTVBss in the presence or absence of dex. Plasmids were cleaved at mutation sites with *Eco*RI prior to introduction into cells.

[b] Recombination frequencies for each experiment were calculated as described in Materials and Methods.

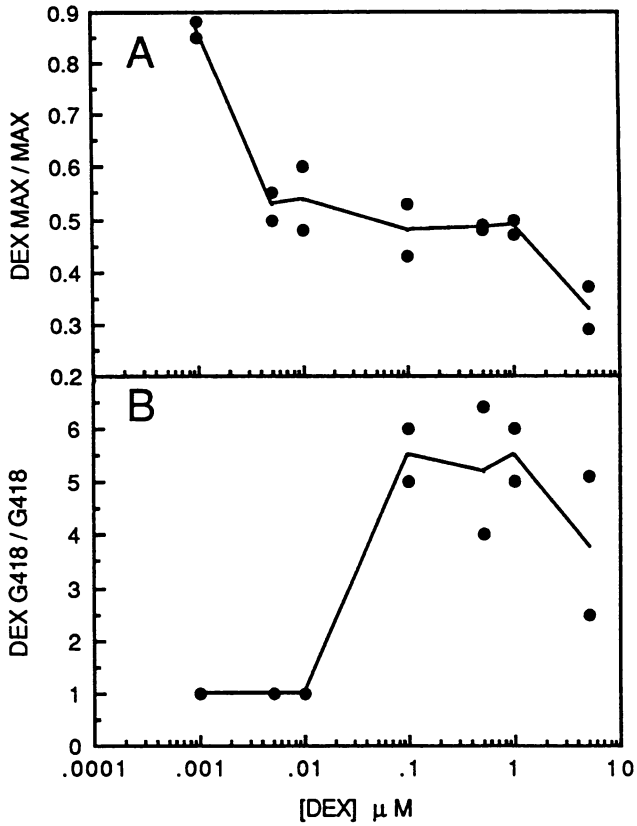


FIG. 5. Effect of dex concentration on transfection and recombination. (A) Dex was added at concentrations of 0.001 to 5.0 μM. The ratio of the MAX$^r$ frequency with dex treatment to MAX$^r$ frequency without dex treatment (DEX MAX/MAX) is plotted for each concentration of dex. Results for two determinations at each concentration tested are shown. (B) Ratios of G418$^r$ frequency with dex treatment to G418$^r$ frequency without dex treatment (DEX G418/G418) are given as in panel A.

To detect less frequent events, the experiment was repeated with a pool of about 1,000 MAX$^r$ colonies. The results parallel those obtained with independent MAX$^r$ colonies. Colony counts of replicate dilutions plated in each medium were as follows: nonselective, 652; MAX, 633; dex plus MAX, 612; G418, 194; and dex plus G418, 129. Thus, 33% of the pooled MAX$^r$ cells were also G418$^r$, and dex treatment did not increase the number of G418$^r$ cells. These results are consistent with the observed dex-induced stimulation of recombination occurring prior to stable integration. However, in these experiments plasmids may integrate in a way that precludes subsequent productive recombination events. Thus, we cannot rule out the possibility that transcription also stimulates recombination between integrated genes.

**Effects of various dexamethasone concentrations on transfection and recombination.** To further characterize the properties of dex-induced recombination, various concentrations of dex were added after electroporation. In these experiments, *Eco*RI-cleaved pMSGneo(Bal)-R and pMSGneo (Bss)-R were used as recombination substrates. Immediately following electroporation, dex was added to final concentrations ranging from 0.001 to 5.0 μM. Twenty-four hours later, dex medium was replaced with selective (MAX or G418) medium. The results are summarized in Fig. 5. There was no change in either the increase in recombination between *neo*

genes or the reduction in MAX$^r$ transfection frequency with dex concentrations from 0.1 to 5.0 μM. No stimulation of recombination was seen with dex concentrations of 0.01 μM or less, but dex MAX$^r$ frequencies did not approach MAX$^r$ frequencies until the dex concentration was substantially lower (0.001 μM). Thus, SV40 *gpt* transfection is sensitive to lower dex concentrations than that required to stimulate recombination between MMTV *neo* genes.

## DISCUSSION

**Transcription-induced homologous recombination.** Previous studies have shown that transcription stimulates homologous recombination in yeast cells (18, 45, 48; J. Nickoloff, unpublished observations). Transcription stimulates both reciprocal and nonreciprocal (gene conversion) events between duplicated regions in yeast cells, but only when both regions are transcribed (48). Mating-type interconversion in yeast cells is mediated by a specific gene conversion event initiated by a double-strand break (DSB) in *MAT*, an expressed copy of a triplicated region (reviewed in reference 13). Klar et al. (20) suggested that the transcriptional activity of *MAT* may regulate initiation of this recombination event.

Immunoglobulin gene rearrangement is another specialized recombination system with similarities to *MAT* switching. For example, it is thought that each system uses a specific recombinase to introduce DSBs in the recombining loci: the HO nuclease in yeast cells (19, 22) and, possibly, the *RAG1* gene product in mammalian cells (1, 39). Furthermore, it has been proposed that transcription may play a role in regulating the initiation of recombination in both systems (1, 20). In immunoglobulin gene rearrangement, it has been suggested that transcription could lead to increased accessibility of a target sequence to a recombinase (1). An alternative explanation is that a process that increases accessibility to RNA polymerase (i.e., changes in chromatin structure) also increases accessibility to a recombinase (1).

In this study, we have demonstrated that transcription stimulates homologous recombination between transfecting plasmids in mammalian cells. Dex-induced transcription of heteroallelic MMTV *neo* genes stimulates recombination sixfold over noninduced levels. *neo* alleles under SV40 promoter control recombine at a frequency similar to that observed for alleles under MMTV promoter control in the presence of dex (about $3.5 \times 10^{-4}$), but these MMTV *neo* alleles recombine at a rate about sixfold lower in the absence of dex ($6.4 \times 10^{-5}$) (Tables 1 and 2). Furthermore, both recombination levels and transcription levels of SV40 promoter-driven genes are reduced in the presence of dex (Table 2 and Fig. 2). Thus, there is a good correlation between transcriptional activity and the level of homologous recombination for given constructs.

Our results indicate that the increased recovery of G418$^r$ recombinants with dex is not due to increased expression of single or a few *neo*$^+$ genes expressed at insufficient levels in the absence of dex. In fact, dex treatment leads to increased *neo*$^+$ copy numbers in G418$^r$ transfectants (Fig. 4). Dex-induced stimulation of recombination is an MMTV promoter-dependent phenomenon and was detected only when DSBs were introduced at mutation sites. The requirement for DSBs suggests that transcription alone may not be sufficient to stimulate recombination in mammalian cells. It is possible, however, that transcription of both substrates might inhibit recombination (see below) or that the effects of transcription in the absence of DSBs may be too small to measure in this system.

Another question is whether the observed effects are due to events occurring before or after integration. Because of difficulties encountered during transient expression assays, we could not demonstrate MMTV promoter activity prior to integration, and therefore we cannot state unequivocally that transcription stimulates recombination prior to integration. However, we did not observe any effects when dex was added to stably transfected cell lines, a result that is consistent with this idea. A less likely explanation is that dex induces recombination soon after integration, but that during culture expansion the integrated copies become refractory to dex-induced recombination.

The results with substrates cleaved at mutation sites suggest that transcriptional enhancement of recombination may require that only one of the two recombining regions be transcribed. This idea arises from considerations of recombination models, including that proposed by Lin et al. (25–28), and the DSBR model (44). According to the model of Lin et al., recombination between two substrates occurs when single-stranded regions (exposed by exonuclease activity) anneal in a segment of shared homology, a nonconservative reciprocal recombination mechanism (7, 27, 28). This model predicts that pMSGneo substrates cleaved at mutation sites produce *neo*$^+$ recombinants carrying the 5′ end of *neo* and the MMTV promoter from pMSGneo(Bss) and the 3′ end of *neo* from pMSGneo(Bal). Since *Eco*RI cleavage of pMSGneo(Bal) separates the 3′ region participating in the reaction from the MMTV promoter, only the 5′ region is predicted to be transcriptionally active.

An alternative model, the DSB repair model (44), has also been invoked to account for mammalian recombination (5, 6, 15, 40, 46). In this model, recombination is initiated by a DSB, the ends invade homologous DNA and prime DNA synthesis, with the homologous DNA acting as the template. Thus, the DSB, or double-strand gap, is repaired in the initiating molecule (the recipient of information) with information from the homologous molecule (the donor), producing either reciprocal or nonreciprocal products (44). This model allows for but does not require recombination to be conservative.

Conclusions reached regarding the transcriptional activity of substrates when the DSB repair model is used are different from those reached by using the model of Lin et al. (25–28). During DSB repair, either molecule can initiate the reaction; since transcription would end at the site of the DSB, only one of the two initiating (invading) ends would be transcriptionally active. The transcriptional state of the noninitiating (donor) molecule, however, would depend on which of the substrates initiated the reaction. If *Eco*RI-digested pMSGneo(Bss) initiated the reaction, the donor region would be inactive. In the opposite case, the donor region would be active. We are currently exploring the relationship(s) between transcriptional activity and recombination in an effort to shed light on these recombination mechanisms.

Interestingly, the idea that only one of the two substrates needs to be transcribed to stimulate recombination is consistent with the model accounting for transcriptional enhancement of recombination proposed by Ikeda and Matsumoto (14). Those authors suggested that the strand displaced during transcription might invade a homologous duplex, promoting recombination. This model does not require that both duplexes be transcribed. In fact, if both duplexes were transcribed, identical strands would be displaced from both duplexes, and the displaced strands might be less likely to invade a more stable RNA-DNA duplex than a nontran-

scribed and less stable DNA-DNA duplex. This model, therefore, might account for the absence of dex-enhanced recombination between circular substrates.

**Effects of dex on transfection with genes under SV40 or MMTV promoter control.** While dex increased homologous recombination between MMTV *neo* alleles on pMSGneo derivatives, it reduced the transfection frequency of the linked *gpt* gene driven by the SV40 early promoter (Table 1). Dex also reduced transfection frequencies of pSV2neo and pSV2gpt (data not shown) and reduced the recombination frequency in SVBal and SVBss crosses (Table 2). These reductions could be due to any of a number of factors, including reductions in cell viability, reduced integration efficiency, or specific interactions between the glucocorticoid receptor-dex complex and the SV40 promoter.

In control experiments, when dex was present for either 1 or 11 days, it was found to have no effect on cell viability in nonselective medium, and dex did not affect the viability of preselected $MAX^r$ transfectants grown in MAX medium (data not shown). Dex did not reduce the transfection efficiency of the MMTV *neo* gene linked to the SV40 *gpt* gene in pMSGneo. Since dex does not appear to affect cell viability or transfection efficiency, it would appear that dex must interact in some deleterious manner with the SV40 promoter. This deleterious interaction might produce nonfunctional *gpt* integrants (e.g., with promoter and gene sequences separated), accounting for the lower transfection frequency of the SV40 *gpt* gene relative to the linked MMTV *neo* gene in pMSGneo. We note that the DNA-binding domain of the glucocorticoid receptor and the SV40 T antigen share amino acid sequence homology (3), and T antigen binds to both the SV40 origin of replication and the nearby early promoter transcription start site (10). We are currently investigating whether dex reduces transfection through specific interactions with the SV40 promoter by repeating these experiments with genes driven by promoters from other sources. An alternative explanation is that dex reduces SV40 promoter transcription and that this reduction leads to reduced transfection efficiency of SV40 promoter-driven genes. While dex-induced transcription from the MMTV promoter might suppress transcription of a linked SV40 promoter (11, 12, 17), such suppression cannot account for dex-mediated reductions in pSV2neo and pSV2gpt transfection frequencies and dex-mediated reductions in recombination frequencies with pSV2neo derivatives.

**Conclusion.** How does transcription stimulate recombination? Ikeda and Matsumoto (14) proposed that single-strand regions displaced during transcription invade homologous DNA, thereby stimulating recombination. Work by Voelkel-Meiman et al. (48) showed that both duplicated regions must be transcribed to stimulate recombination in yeast cells. Our results and those of other laboratories indicate that transcription may play a role in a variety of recombination processes. It is possible, considering the widespread nature of transcription-induced recombination, that a variety of mechanisms are involved. Further studies with other regulated promoters, such as metallothionine gene promoters, will extend our understanding of these processes in mammalian cells.

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