The Assembly of Progesterone Receptor-hsp90 Complexes Using Purified Proteins*

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The progesterone receptor can be reconstituted into hsp90-containing complexes in vitro, and the resulting complexes are needed to maintain hormone binding activity. This process requires ATP/Mg\(^{2+}\), K\(^+\), and several auxiliary proteins. We have developed a defined system for the assembly of progesterone receptor complexes using purified proteins. Five proteins are needed to form complexes that are capable of maintaining hormone binding activity. These include hsp70 and its co-chaperone, hsp40, the hsp70/hsp90-binding protein, Hop, hsp90, and the hsp90-binding protein, p23. The proteins Hip and FKBPs were not required for this in vitro process even though they have been observed in receptor complexes. Each of the five proteins showed a characteristic concentration dependence. Similar concentrations of hsp70, hsp90, and p23 were needed for optimal assembly, but hsp40 and Hop were effective at about 1/10 the concentration of the other proteins, suggesting that these two proteins act catalytically or are needed at levels similar to the receptor concentration. ATP was required for the functioning of both hsp70 and hsp90. The binding of hsp70 to the receptor requires hsp40 and about 10 \(\mu M\) ATP; however, hsp90 binding appears to occur subsequent to hsp70 binding and is optimal with 1 mM ATP. A three-step model is presented to describe the assembly process.

When extracted from tissue cytosol, receptors for progesterone (PR), glucocorticoid (GR), and other steroids exist in heteromeric complexes containing heat shock protein 90 (hsp90) and several additional proteins (1–3). Recent information on the assembly of these complexes has been gained mainly through the use of an in vitro system consisting of immune-isolated progesterone (4) or glucocorticoid (5) receptor incubated in rabbit reticulocyte lysate. The formation of receptor complexes in this system is dependent upon ATP hydrolysis, the ions Mg\(^{2+}\) and K\(^+\), and the participation of a number of proteins (6, 7). When assembled in vitro, the mature avian progesterone receptor complex closely resembles that obtained from oviduct cytosol and contains hsp90, any one of three immunophilins, and a 23-kDa phosphoprotein, p23. It also contains variable sub-stoichiometric amounts of hsp70. The immunophilins include the cyclosporin A-binding protein, CyP40, and two FK506-binding proteins, FKBP51 and FKBP52. Antibody inhibitor studies or depletion and reconstitution experiments indicate that hsp70 (6, 8), Hop, an intermediate in complex assembly (9), and p23 (10, 11) are essential for the formation of hsp90 complexes, but the actual roles of these and other proteins are still unclear.

An intermediate in PR complex formation has been identified that contains submaximal amounts of hsp90, substantially more hsp70 than in the mature complex, and two additional proteins, Hop (p60) and Hip (p48) (6, 12). This complex does not contain immunophilins or p23. Hop (hsp-organizing protein) is a 60-kDa stress-related protein that binds to both hsp70 and hsp90 (9, 13–15). Hip (hsp70-interacting protein) is also known to bind to hsp70 (16, 17). This intermediate complex has been shown in time course studies of PR assembly, and it is the predominant form when the ATP level is suboptimal (6, 12) and when further assembly is blocked by the benzoquinone ansamycin, geldanamycin (18, 19), which binds to the ATP-binding site on hsp90 and blocks some of its functions (20–23). Thus, the assembly of PR complexes includes at least two steps and a major intermediate complex.

To gain further understanding on the mechanism of receptor complex assembly requires the use of more defined, purified systems. Toward this end, Pratt and co-workers (24, 25) have successfully used purified rabbit hsp70 and hsp90 plus recombinant Hop and p23 to assemble complexes of the glucocorticoid receptor. More recently, they have also shown a requirement for hsp40 (DnaJ homolog) in this system (26). Their results indicate that glucocorticoid receptor complexes capable of binding hormone can be assembled using only hsp70, hsp40, hsp90, and Hop and that p23 is not essential but it stabilizes the receptor complexes to dramatically enhance hormone binding activity. We now report the assembly of progesterone receptor complexes using highly purified preparations of hsp70, hsp90, hsp40, Hop, and p23. We describe the conditions needed to assemble individual proteins into the complex to generate the hormone-binding state of the receptor.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal (IgG) antibody PR22 against the avian PR has been described previously (27). GA was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. GA was dissolved in dimethyl sulfoxide (Me\(_2\)SO) and used at the concentrations indicated.

Protein Purification—Human hsp908 was overexpressed in SF9 cells as described by Alnemri and Litwack (28). The protein was purified from cytosol extracts to >99% purity by chromatography on columns of DEAE-cellulose, heparin-agarose, and Mono Q as described previously (29). The purified hsp90 was stored at −70 °C in 10 mM Tris-HCl, 100 mM Me\(_2\)SO, 20% glycerol.
mm KCl, 1 mM EDTA, and 10% glycerol, pH 7.4.

hs70 was prepared by the overexpression of human hs70 in SF9 cells using the system of Alnemri and Litwack (28). The purification was as described previously for avian hs70 (30). Cell lysates were fractionated by DEAE-cellulose column chromatography followed by ATP-agarose column chromatography. The preparation was precipitated using ammonium sulfate (75% saturation), and the redissolved hs70 was fractionated by 16/60 Superdex 200 FPLC. Only the monomer peak of hs70 was used. The preparation was approximately 97% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

Human Hop expressed in bacteria was prepared essentially as described previously (31). Bacterial lysates were fractionated by DEAE-cellulose chromatography followed by hydroxylapatite column chromatography. Additional purification was achieved by fractionating the pool from hydroxylapatite on a Mono Q FPLC column (10/10, Amersham Pharmacia Biotech) that was eluted with a linear gradient of 0–0.5 M KCl. The fractions containing Hop were pooled, dialyzed into 10 mM Tris-HCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.5, and stored at −70 °C. The preparation was approximately 94% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

A bacterial expression system for Ydj1p was generously supplied by Dr. Avrom Caplan and has been described previously (32). Bacterial lysates were fractionated by DEAE-cellulose column chromatography followed by hydroxylapatite column chromatography. The preparation was approximately 80% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis. Human HDJ-2 was expressed in bacteria and purified as described previously (31). Bacterial lysates were fractionated by DEAE-cellulose chromatography followed by phenyl-Sepharose chromatography followed by hydroxylapatite column chromatography. The preparation was approximately 80% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

Human HDJ-2 was expressed in bacteria and purified as described previously (32). The soluble fraction of bacterial lysate was fractionated by hydroxylapatite column chromatography followed by hydroxylapatite column chromatography. The preparation was approximately 94% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

RESULTS

When the avian PR is isolated on antibody resin and stripped of its associated proteins, its hormone binding domain becomes very unstable (12). Hormone binding activity can be maintained for several hours when in an ice bath, but it is rapidly lost at elevated temperatures. However, binding activity can be maintained at elevated temperatures or restored from the inactive state by reconstitution of the PR-hsp90 complex in reticulocyte lysate (12). An example of this is shown in Fig. 1. The A and B forms of the PR were first isolated on antibody resin, and endogenous hs90 and other associated proteins were removed by treatment with 0.5 M KCl (lane 1). Identical PR samples were then treated for 30 min at 30 °C in rabbit reticulocyte lysate to restore the complex with hs90 (lane 2) or to make an intermediate complex by either limiting the ATP by omitting the ATP-regeneration system (lane 3) or by treatment with the hs90 inhibitor, geldanamycin (GA, lane 4). Reversal of GA inhibition could be accomplished by isolating the PR complex and incubating it with fresh lysate (lane 5). Fig. 1B shows the hormone binding activities of these preparations using a saturating concentration of [3H]progesterone. A major portion of binding activity can be maintained at 30 °C, but this is not possible when complex formation is compromised by omitting the ATP regeneration system or by treating with GA. Therefore, loss of hormone binding correlates with conditions that decrease hs90 association in favor of hsp70 binding. In preliminary studies, we were able to reverse the GA-inhibited state (lane 4) by an incubation of the PR complex with three purified proteins, hs90, hs70, and p23 (results not shown).

In this effort we extended to develop a reconstitution system entirely composed of purified proteins as described below.

In developing a defined system, we have used both the analysis of protein composition and the measurement of hormone binding to assess PR complex formation. Fig. 2 illustrates the loss of hormone binding activity upon incubation at 30 °C under various conditions. When in the absence of other proteins, the PR loses hormone binding activity progressively over a 30-min period. No stabilization of binding activity was observed when the PR was incubated with hs70, ATP, and the yeast hs40 homolog, YDJ-1. However, the third condition (complete) included ATP plus the five proteins, hs70, YDJ-1, Hop, hs90, and p23 (results not shown).

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The extent to which binding activity is maintained is somewhat variable within the range of 50–80% of the activity at the start of the experiment. Fig. 2B shows the protein composition of PR isolated after 30 min incubation under the three conditions. In all cases, the PR-A and PR-B receptor forms are maintained with no loss due to degradation. When all proteins are present, a multi-protein complex including hs90, hs70, Hop, and p23 (not shown) is formed with proportions that resemble those of native PR complexes (6, 12). When only hs70 and YDJ-1 are present, they readily bind to the receptor, but they are not sufficient for providing a hormone-binding structure.

Although hormone binding is lost in the presence of hs70 and YDJ-1, these proteins may protect the PR against further denaturation. To test this, two sample sets containing PR-bound resin and 5 mM ATP, with or without hs70 and YDJ-1, were incubated at 30 °C for 30 min to destabilize the receptor. The samples were then supplemented to contain all five chap-
erone proteins and incubated at 30 °C for the times indicated (Fig. 3). A significant amount of hormone binding activity could be restored to the 30 °C-treated PR by a second 30 °C incubation in the presence of all five chaperones. The presence of hsp70 and YDJ-1 from the beginning enhanced the recovery of hormone binding and appeared to protect a portion of the PR from becoming irreversibly denatured. This suggests that although hsp70 and YDJ-1 cannot maintain hormone binding activity, they hold the receptor in a state that can be more readily restored to native structure when all five proteins are present.

**FIG. 1.** The reconstitution of various PR complexes in rabbit reticulocyte lysate. A, Coomassie-stained SDS-PAGE separations of PR complexes. B, hormone binding activities of various PR complexes reconstituted with rabbit reticulocyte lysate. Avian PR was isolated from oviduct cytosol using antibody PR22 bound to protein A-Sepharose. The PR stripped (Str) in 0.5 M KCl is shown in lane 1. Samples identical to lane 1 were reconstituted (Rec) into complexes by incubation in rabbit reticulocyte lysate (r.l.) for 30 min at 30 °C in the presence (lane 2) or absence (lane 3) of an ATP-regenerating system (RS). The sample in lane 4 was reconstituted into an intermediate complex by incubation in rabbit reticulocyte lysate plus an ATP-regenerating system for 30 min at 30 °C in the presence of 10 μg/ml geldanamycin (GA). The PR complex shown in lane 4 was incubated with fresh reticulocyte lysate again for 1 h at 30 °C without GA (lane 5). A, receptor forms A and B along with associated proteins are indicated on the left as well as antibody heavy (HC) and light (LC) chains. Molecular weight markers are indicated on the right including phosphorylase b, 97,000; bovine serum albumin, 68,000; ovalbumin, 43,000; lactate dehydrogenase, 36,000; carbonic anhydrase, 29,000 and trypsin inhibitor, 20,400.

**FIG. 2.** The influence of proteins on the hormone binding activity of PR at 30 °C. A, samples of stripped PR adsorbed to antibody resin were incubated at 30 °C for the times indicated. The samples contained incubation buffer plus 5 mM ATP (triangles); 20 μg of hsp70, 2 μg of YDJ, and 5 mM ATP (squares), or the complete system with 20 μg of hsp70, 2 μg of YDJ, 5 μg of Hop, 20 μg of hsp90, 5 μg of p23, and 5 mM ATP (circles). After incubation, the samples were analyzed for hormone binding activity and for composition by SDS-PAGE. B shows the Coomassie-stained gel of the stripped PR before incubation (lane 1) and samples after incubation at 30 °C for 30 min in the complete system (lane 2), plus hsp70 and YDJ (lane 3), or without added proteins (lane 4).

**FIG. 3.** The reconstitution of various PR complexes in rabbit reticulocyte lysate. A, samples of stripped PR adsorbed to antibody resin were incubated at 30 °C for the times indicated. The samples contained incubation buffer plus 5 mM ATP (triangles); 20 μg of hsp70, 2 μg of YDJ, and 5 mM ATP (squares), or the complete system with 20 μg of hsp70, 2 μg of YDJ, 5 μg of Hop, 20 μg of hsp90, 5 μg of p23, and 5 mM ATP (circles). After incubation, the samples were analyzed for hormone binding activity and for composition by SDS-PAGE. B shows the Coomassie-stained gel of the stripped PR before incubation (lane 1) and samples after incubation at 30 °C for 30 min in the complete system (lane 2), plus hsp70 and YDJ (lane 3), or without added proteins (lane 4).

**FIG. 4.** The reconstitution of various PR complexes in rabbit reticulocyte lysate. A, samples of stripped PR adsorbed to antibody resin were incubated at 30 °C for the times indicated. The samples contained incubation buffer plus 5 mM ATP (triangles); 20 μg of hsp70, 2 μg of YDJ, and 5 mM ATP (squares), or the complete system with 20 μg of hsp70, 2 μg of YDJ, 5 μg of Hop, 20 μg of hsp90, 5 μg of p23, and 5 mM ATP (circles). After incubation, the samples were analyzed for hormone binding activity and for composition by SDS-PAGE. B shows the Coomassie-stained gel of the stripped PR before incubation (lane 1) and samples after incubation at 30 °C for 30 min in the complete system (lane 2), plus hsp70 and YDJ (lane 3), or without added proteins (lane 4).
Comparing the results obtained using either YDJ-1 or HDJ-2, we have used YDJ-1 for most of our studies because it was the first cytoplasmic hsp40 that was readily available. It is somewhat more consistent than HDJ-2 in our hands.

The dependence upon each protein in the system for PR complex assembly was tested as shown in Fig. 5. Lane C shows the binding activity of PR kept in ice, and the 2nd lane (T) shows the activity in the complete system after 30°C for 30 min. Individual proteins were omitted from the system which caused a substantial loss of binding activity in all cases. Omis-sion of hsp70 consistently resulted in the greatest loss of binding activity. This figure also shows the system to be completely dependent on ATP and that the addition of GA, a competitive inhibitor for the ATP-binding site on hsp90, inhibits in this system. An ATP regeneration system was found not to be necessary in this system with purified proteins.

The relationship between hsp70 and hsp90 is shown more clearly in Fig. 6. In this experiment, the ATP concentration was varied, and the complexes produced were analyzed for hormone binding and composition. hsp70 binding is greatest with 10 μM ATP, whereas hsp90 binding and the development of hormone binding activity are optimal with an ATP concentration of 0.5 to 1 mM. Thus, the ATP requirement for hsp70 binding is much lower than for hsp90 binding, and the hormone binding activity of PR correlates with the binding of hsp90. As the binding of hsp90 progresses, the binding of hsp70 diminishes suggesting a reciprocal relationship between these two chaperones. The correlation between hsp90 binding and the appearance of hormone binding activity is also illustrated in Fig. 7 where complex formation is measured in relation to hsp90 concentration. The binding of p23 (not shown) also correlates with hsp90 binding.

We looked more closely at the interaction between hsp90 and p23 in Fig. 8. In this experiment, PR was first incubated for 30 min at 30°C in the presence of hsp70, YDJ-1, Hop, and ATP which caused a loss of hormone binding activity to near background. This was followed by a second 30°C incubation after adding hsp90, p23, or both. It is clear that both hsp90 and p23 are required to obtain a significant recovery of hormone binding.

In one experiment, the ATP concentration was varied, and the complexes produced were analyzed for hormone binding and composition. hsp70 binding is greatest with 10 μM ATP, whereas hsp90 binding and the development of hormone binding activity are optimal with an ATP concentration of 0.5 to 1 mM. Thus, the ATP requirement for hsp70 binding is much lower than for hsp90 binding, and the hormone binding activity of PR correlates with the binding of hsp90. As the binding of hsp90 progresses, the binding of hsp70 diminishes suggesting a reciprocal relationship between these two chaperones. The correlation between hsp90 binding and the appearance of hormone binding activity is also illustrated in Fig. 7 where complex formation is measured in relation to hsp90 concentration. The binding of p23 (not shown) also correlates with hsp90 binding.

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We have titrated the amount of each protein required in this reconstitution system. Table I lists the amount needed for maximum activity and the concentration at half-maximum. These data clearly illustrate two points. First, very comparable molar amounts of hsp70, hsp90, and p23 are needed. Under our conditions, these proteins are in about 10–20-fold excess over the PR concentration. The second point is that YDJ-1 and Hop are only required in 1/5 to 1/10 the amount of the other proteins. This suggests that they are either catalytic in their activities or that their requirements more closely match the PR concentration. It should be noted that an excess of YDJ-1 is detrimental, causing a marked reduction in the development or maintenance of hormone binding activity. This inhibitory effect has not been observed with the other proteins in the system.

**DISCUSSION**

Our results show that the native state of the PR complex, as indicated by hormone binding capacity and by composition, can be maintained in vitro at 30 °C by the use of the following five additional proteins: hsp70, hsp40, Hop, hsp90, and p23. Once the hormone binding activity is lost, it can be recovered substantially by treatment with these proteins. These results are in agreement with the studies of Pratt and co-workers (24–26) on the reconstitution of glucocorticoid receptor complexes. Our study provides additional information on the amounts of protein required, the proportions of proteins in the complex, and some of the interrelationships among the proteins.

Our studies suggest that PR complex formation proceeds through three primary steps as illustrated in Fig. 9. Initially, perhaps because of some structural instability in the hormone binding domain, the receptor is recognized by hsp70 and hsp40 (step 1). This may involve the rapid but very reversible binding of hsp70-ATP which is then converted to PR-hsp70-ADP through the YDJ-1-dependent hydrolysis of ATP by hsp70. Since the binding of hsp70 (above background) in PR is strictly dependent on YDJ-1 or HDJ-2 and ATP, it is likely that only the tighter binding of hsp70-ADP is observed because it withstands the procedure used for complex isolation.

The recruitment of Hop-hsp90 to the PR complex (step 2) would occur once bound hsp70-ATP is converted to hsp70-ADP. Previous studies have shown that Hop can bind hsp70-ADP but not hsp70-ATP (12, 14). At this stage, the hsp90 would be either nucleotide-free or bound with ADP since hsp90 treated with ATP and molybdate has a low affinity for Hop (14). In this intermediate, the receptor might be shared by hsp70 and hsp90 or passed back and forth until its properties change to a state of preferential binding to hsp90. hsp90 can then be converted to the ATP-bound conformation which is stabilized and perhaps facilitated by the binding of p23. The PR acquires hormone binding activity coincident with its conversion during step 3. Hop and hsp70 appear to be released at this step; however, at least in vitro, this release is never complete. This may be because step 3 does not proceed very efficiently in vitro, or there may be a continued need for some hsp70 interaction to maintain the final hsp90 complex. The mechanism of hsp70 release is still unclear, but this is likely to involve the conver-
concentration of about 1 mM. This higher ATP level may serve the last hsp90-mediated step but at a much higher

23

90

), and finally with p23 (29). It may also facilitate the generation of excess hsp70-ADP which may both extend the binding of hsp70 to PR and increase the requirement for Hop.

The model in Fig. 9 generally agrees with previous models (1–3). However, most previous models have emphasized multi-protein complexes as operational units of these proteins. Two types of hsp90 complexes are abundant in cell extracts, one with Hop and hsp70 and another with p23 and immunophilins (1–3). These complexes are not static, and the interactions among components are influenced by nucleotide binding as discussed above. The three steps in Fig. 9 have not been demonstrated as isolated events, and they may imply a greater separation of events than occurs in the cell. Since pre-formed chaperone complexes exist, they are likely to be operational in this process. For example, one could consider the possibility that all five of the proteins studied here relate closely to a complex or foldosome (44) that is mobile and somewhat variable in composition depending on its stage of activity.

The role of p23 in the last step of PR complex assembly is still unclear. In their studies with the glucocorticoid receptor, Pratt and co-workers (25) have provided evidence that p23 acts to stabilize and increase the amount of GR complexes in the hormone-binding state, but a significant amount of hormone binding complex can be produced in the absence of p23. That p23 is not essential is also indicated in a recent report by Bohm (42) using a yeast system containing GR and a reporter gene. He showed that deletion of the p23 gene of Saccharomyces cerevisiae had a minimal effect on GR action, but it did sensitize the GR to inhibition by the GA-related drug, macbescin II (42). Another report by Fang et al. (43) showed that the deletion of yeast p23 resulted in only a mild reduction in cell growth or the hsp90-dependent activities of the tyrosine kinase v-Src and the androgen receptor. Our results show a marked dependence upon p23 for the generation of hormone binding activity. It is possible that the dependence on p23 would be diminished in the presence of hormone. However, in preliminary experiments where the hormone was included during the 30 °C incubation of PR with chaperones, this resulted in only a slight increase in hormone binding (not shown). It is still quite possible that some contributing factors are missing from our minimal system (see below), and any deficiencies may enhance the dependence on p23.

The present experimental system should be considered as a minimal system that is probably still incomplete. Two types of proteins, Hip and the immunophilins, are missing even though these have been clearly observed in steroid receptor complexes. In rabbit reticulocyte lysate, Hip binds to PR complexes at the intermediate stages of steps 1 and 2 (17). Hip has been shown to bind to hsp70 and to stabilize the ADP-bound state of hsp70 (16). Thus far, we have not observed any influence of Hip on the reconstitution system described here. Perhaps additional conditions or factors are needed to reveal a role for Hip in this system.

Since immunophilin binding does not occur until the final step in assembling PR complexes (12), they may not be important to the assembly process per se but may fulfill a role subsequent to assembly. Little is known about the potential functions of FKBP51, FKBP52, and CyP-40. Pratt and co-workers (38, 39) have provided evidence that FKBP52 may be involved in the nuclear transport of steroid receptors. The immunophilins, having peptidyl-prolyl isomerase activity, could also interact with the receptor to modulate its conformational state either before or after the binding of hormone. Two recent reports relate to this latter possibility. The peptidyl-prolyl isomerase, Pin 1, interacts with and regulates the activities of

### Table 1: Concentration of ingredients for PR assembly

| Component | Maximum | Half-maximum |
|-----------|---------|--------------|
| PR (~0.05 μM) | 1500 μM | 300 μM |
| ATP | 1.4 μM | 0.6 μM |
| hsp70 | 0.8 μM | 0.2 μM |
| p23 | 1.3 μM | 0.3 μM |
| YDJ-1 | 0.2 μM | 0.06 μM |
| Hop | 0.08 μM | 0.02 μM |

*Estimates were averaged from two experiments for the concentrations required for maximum assembly and for half of the maximum.

The hsp90 concentration is calculated as a homodimer.

### Fig. 9. A three-step model for the assembly of PR complexes.

The receptor (R) is shown to interact with hsp70 (70), followed by recruitment of Hop and hsp90 (90), and finally with p23 (23).
several mitotic proteins (40). It selects these proteins and modifies their conformation by binding to a phosphoserine-proline motif. In another study, CyP-40 was shown to bind the transcription factor c-Myb resulting in an inhibition of its activity (41). In any case, we have tested one immunophilin, FKBP52, and it does not affect PR complex formation under our conditions.

Thus, several important questions remain to be answered concerning the hsp70, hsp90 pathway for the assembly and functioning of target protein complexes. The present system provides a defined framework for future mechanistic studies on the functioning of this chaperoning pathway and for elaborating on additional components and functions of this process.

Acknowledgments—We thank Sherry Linander for assistance in manuscript preparation. SF9 cell growth, treatment, and harvesting were conducted by Dean Edwards and Kurt Christenson at the University of Colorado Cancer Center Tissue Core. We thank Gerald Litwack, Emad Alnemri, Avram Caplan, and Seishi Kato for generously supplying DNA reagents.

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